OZONE BASED TREATMENTS FOR INACTIVATION OF
SALMONELLA ENTERICA SEROVAR ENTERITIDIS IN SHELL EGGS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy
in the Graduate School of The Ohio State University

By
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2010
Abstract

Illnesses due to ingestion of *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*Salmonella* Enteritidis) increased steadily and inexplicably throughout the 1980s. Numerous risk assessment studies have demonstrated that the number one risk factor for *Salmonella* Enteritidis illness is the consumption of eggs or egg-containing products. As this serious health threat persisted throughout the 1990s, increased research efforts elucidated the complexity of the issue at hand. Eggs may be contaminated during or after lay, leading to the presence of cells on the egg shell, in the egg contents (in albumen or yolk), or both. While selected technologies have proven efficacious in reducing external and/or internal *Salmonella* Enteritidis contamination in whole eggs, products produced using these methods are far from ideal. Both extended heat treatment and application of gamma irradiation have been demonstrated to greatly reduce *Salmonella* Enteritidis contamination, but both processes also compromise the quality of treated eggs. A process that can combine safety with the production of a high-quality product is needed.

The objectives of this research were: (i) to evaluate the efficacy of heat and ozone combination treatment against *Salmonella* Enteritidis, inoculated onto the vitelline membrane using pilot-scale equipment and to verify the acceptable quality of treated eggs; (ii) to develop a protocol sufficient to pasteurize eggs inoculated with *Salmonella* Enteritidis in the yolk; and (iii) to examine the chemical changes, physical quality and
functionality of eggs treated with a pasteurization process. The effect of inoculation and incubation procedure on process lethality was also investigated.

Shell eggs were inoculated with *Salmonella* Enteritidis near the vitelline membrane (~10^5 CFU/egg). Inoculated eggs were subjected to heat (57°C for 21 minutes), gaseous ozone (vacuum of 67.5 kPa broken with gaseous ozone at maximum concentration of ~140 g m^-3 and pressure of 184 – 198 kPa for 40 minutes) or both. Following treatment, eggs were analyzed using a modified most probable number (MPN) technique. Ozone alone, heat alone and combination treatments resulted in a reduction of *Salmonella* Enteritidis of 0.11, 3.1 and 4.2 log CFU/egg, respectively.

The quality of eggs subjected to this combination treatment was verified in a six week storage study. Treated and untreated eggs were held at 4 or 25°C and were subjected to weekly quality testing. At 4°C, treated and untreated eggs retained similar quality throughout storage. At 25°C, albumen quality, as defined by Haugh units and albumen pH, was maintained significantly better in treated eggs than in untreated samples.

Process parameters were fine-tuned in the design of a treatment capable of pasteurizing eggs inoculated with *Salmonella* in the yolk. In this study, eggs were inoculated within the yolk and incubated overnight at 30°C, allowing cells to multiply to high population (representing the worst-case-scenario for naturally contaminated eggs). Final population of *Salmonella* Enteritidis before treatment was approximately 10^7 CFU g^-1 egg contents. Contaminated eggs were treated with heat in one of several time-temperature combinations or heat followed by ozone (vacuum of 67.5 kPa broken with gaseous ozone at maximum concentration of ~160 g m^-3 and pressure of 184 – 198 kPa
for 60 minutes). All treatments tested resulted in greater than 6 log CFU g⁻¹ reduction in internal Salmonella, but produced eggs varying greatly in visual appeal. From this set of treatments, one was selected for in-depth quality and functionality testing.

Physical quality and functionality of ozone plus heat pasteurized eggs was compared with that of heat pasteurized and untreated eggs over the course of eight weeks of storage, under normal storage (4°C) and temperature abuse (25°C) conditions. Quality of albumen was maintained better in pasteurized eggs than untreated eggs, regardless of storage temperature. No significant differences were observed in yolk quality. While ozone-based pasteurization reduced functionality of albumen compared to untreated eggs, heat pasteurization resulted in the greatest functionality reduction.

Several chemical analyses were performed to better assess the effect of treatments on albumen proteins. Heat plus ozone combination treatment did not damage either ovotransferrin or lysozyme, two of the predominant antimicrobial albumen proteins. Application of ozone did not significantly change the ratio of free sulfhydryl groups to disulfide bonds in albumen. Slight but not significant differences among treatments were observed with FTIR spectroscopy, indicating that the albumen of combination-pasteurized eggs more closely resembled that of untreated eggs than did albumen from heat-pasteurized eggs.

Inoculation/incubation procedures were examined throughout this research and differences in the reduction of Salmonella were observed according to several factors, including the location/depth of inoculum placement, cell density of inoculum, and incubation time and temperature. This research demonstrates that treatment with a
combination of heat and gaseous ozone can be utilized to produce safe, high quality whole shell eggs.
Acknowledgements

I would like to offer my sincerest thanks to my advisor Dr. Ahmed Yousef for his constant support and guidance throughout my time at The Ohio State University. His encouragement and instruction have been invaluable to me as I have strived to become a better scientist and mentor. I can’t imagine having spent this time working for anyone else. To my committee members; Dr. Luis Rodriguez-Saona, Dr. Steve Schwartz and Dr. Jiyoung Lee, thank you for your suggestions and for your patience. Special thanks to Dr. Rodriguez for all his help with FTIR and to Dr. Gonul Kaletunc and Zifei Dai for their assistance with DSC.

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To my mother, Nancy and my sister, Amy; thank you for believing in me unquestioningly and for making me the person I am. Lastly, thanks to my husband James...
for reassuring me when I needed it and just putting up with me when there was nothing else to be done. I love you guys.
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Major field: Food Science and Nutrition
# Table of Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vii</td>
</tr>
<tr>
<td>Vita</td>
<td>ix</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiv</td>
</tr>
<tr>
<td><strong>Chapters</strong></td>
<td></td>
</tr>
<tr>
<td>1. Literature Review</td>
<td>1</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>1</td>
</tr>
<tr>
<td>Eggs</td>
<td>8</td>
</tr>
<tr>
<td>Ozone</td>
<td>36</td>
</tr>
<tr>
<td>References</td>
<td>47</td>
</tr>
<tr>
<td>2. Inactivation of <em>Salmonella enterica</em> serovar Enteritidis in shell eggs by sequential application of heat and ozone and physical quality of eggs treated with this process</td>
<td>63</td>
</tr>
<tr>
<td>Abstract</td>
<td>63</td>
</tr>
<tr>
<td>Introduction</td>
<td>64</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>66</td>
</tr>
<tr>
<td>Results</td>
<td>73</td>
</tr>
<tr>
<td>Discussion</td>
<td>78</td>
</tr>
<tr>
<td>References</td>
<td>80</td>
</tr>
<tr>
<td>3. Development of a heat and ozone combination treatment yielding sufficient inactivation of <em>Salmonella enterica</em> serovar Enteritidis in egg yolk to qualify as a “pasteurization process”</td>
<td>82</td>
</tr>
<tr>
<td>Abstract</td>
<td>82</td>
</tr>
<tr>
<td>Introduction</td>
<td>83</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>87</td>
</tr>
<tr>
<td>Results</td>
<td>94</td>
</tr>
<tr>
<td>Discussion</td>
<td>100</td>
</tr>
<tr>
<td>References</td>
<td>104</td>
</tr>
</tbody>
</table>
4. Quality and functionality of shell eggs processed with a heat and ozone combination process………………………………………………………..…..107
   Abstract………………………………………………………………...107
   Introduction……………………………………………………………108
   Materials and methods…………………………………………………110
   Results………………………………………………………………….115
   Discussion………………………………………………………………128
   References………………………………………………………………132

5. Changes in albumen proteins due to pasteurization of shell eggs with heat or heat-ozone combinations…………………………….…………………………135
   Abstract……………………………………………………………...…135
   Introduction…………………………………………………………….136
   Materials and methods………………………………………………….138
   Results…………………………………………………………………..143
   Discussion………………………………………………………………150
   References………………………………………………………………152

6. Thermal inactivation of *Salmonella enterica* Serovar Enteritidis in whole shell eggs, effect of inoculation site and refrigeration………………………….154
   Abstract…………………………………………………………………154
   Introduction………………………………………………………………155
   Materials and methods…………………………………………………156
   Results…………………………………………………………………..156
   Discussion………………………………………………………………162
   References………………………………………………………………164

Appendix A: Process development……………………………………………………..166

Appendix B: *Salmonella enterica* serovar and strain comparison…………………………..169

Bibliography……………………………………………………………………………182
List of Tables

Table                                                                 Page
1.1. Common foodborne salmonellae and antigenic formulae............................2
1.2. Examples of recent *Salmonella* outbreaks........................................8
1.3. Albumen protein characteristics..........................................................12
1.4. USDA quality standards for egg grading..............................................15
1.5. Recognized egg weight classes..............................................................15
1.6. Summary of requirements for "Lion Quality" eggs (Great Britain).............28
1.7. Pasteurization guidelines for selected egg products..............................32
1.8. Ozone treatment for decontamination of food products..........................45
2.1. Inactivation of *Salmonella* Enteritidis in shell eggs after various treatments and total number of treated samples testing positive for surviving salmonellae..........................................................75
3.1. Summary of heating time and temperature changes of pasteurized eggs......96
3.2. Log reduction of *Salmonella* Enteritidis per gram of egg contents after treatment with heat or heat plus ozone............................................................96
5.1. Lysozyme activity of untreated and pasteurized eggs............................144
5.2. SIMCA interclass distances among treatments.......................................148
6.1. Internal temperature at inoculation site after 1-log reduction of *Salmonella* Enteritidis in inoculated shell eggs....................................................161
A.1. Ozone treatments used for assessment of concentration effect...............168
B.1. Salmonella enterica serovars and strains analyzed..............................174

B.2. Differences in population\textsuperscript{a} of Salmonella serovars during 30 minute heating at 57\textdegree C.................................................................177

B.3. Susceptibility of Salmonella to antibiotics........................................179
List of Figures

Figure                                      Page
1.1. Hen reproductive system.................................9
1.2. Egg structure...............................................11
1.3. Oxidation of indigo trisulfonate by ozone................40
2.1. Benchtop and pilot-scale vessels for ozonation of shell egg........69
2.2. Representation of experimental setup used to treat whole shell eggs with gaseous ozone under pressure...............................70
2.3. Internal egg temperature and water bath temperature during heat treatment of shell eggs..................................................74
2.4. Ozone concentration and pressure inside treatment vessel during treatment of shell eggs..................................................74
2.5. Albumen pH of treated and untreated eggs over six weeks of storage........76
2.6. Yolk index of eggs stored at 25°C for up to six weeks....................77
2.7. Haugh units in eggs stored at 25°C for up to six weeks....................78
3.1. Time/temperature combinations required to produce thermally pasteurized eggs as specified in the US patent 6,632,464.................................87
3.2. Selection of heat treatment steps for heat-ozone pasteurization process.....88
3.3. Custom-made stainless steel rack for heat and ozone treatment of shell eggs.....91
3.4. Growth of Salmonella Enteritidis in egg yolk of inoculated shell eggs incubated at 30°C.................................................................94
3.5. Untreated and heat-pasteurized eggs..............................................95
3.6. Percentage of pasteurized eggs testing positive for *Salmonella* Enteritidis……97

3.7. Eggs pasteurized with heat plus ozone combination treatments………………99

3.8. Reduction of *Salmonella* Enteritidis in eggs subjected to varying heat
treatments followed by ozone treatment……………………………………110

4.1. Adjustable measuring cup used to determine weight of specific volume of
egg white foam……………………………………………………………………114

4.2. Internal egg temperature and water bath temperature during pasteurization
of shell eggs……………………………………………………………………116

4.3. Yolk indices of untreated or pasteurized shell eggs during eight weeks of
storage at 4°C……………………………………………………………………117

4.4. Yolk indices of untreated or pasteurized shell eggs during eight weeks of
storage at 25°C……………………………………………………………………118

4.5. Haugh units of untreated and pasteurized shell eggs during eight weeks of
storage at 4°C……………………………………………………………………120

4.6. Haugh units of untreated and pasteurized shell eggs during eight weeks of
storage at 25°C……………………………………………………………………121

4.7. Albumen pH of untreated and pasteurized shell eggs during eight weeks of
storage at 4°C……………………………………………………………………122

4.8. Albumen pH of untreated and pasteurized shell eggs during eight weeks of
storage at 25°C……………………………………………………………………123

4.9. Albumen turbidity of untreated and pasteurized shell eggs during eight
weeks of storage at 4°C…………………………………………………………125

4.10. Percent overrun of albumen from untreated and freshly pasteurized shell
eggs during 10 minutes of whipping………………………………………………126

4.11. Percent overrun of albumen from untreated and pasteurized shell eggs
stored for 8 weeks at 4°C during 10 minutes of whipping…………………………127

4.12. Percent overrun of albumen from untreated and pasteurized shell eggs
stored for 8 weeks at 25°C during 10 minutes of whipping…………………………128

5.1. Reaction of Ellman's reagent with thiol group to produce colored product……140
5.2. Lysozyme standard curve………………………………………………………141

5.3. Sulphydryl groups in albumen of untreated eggs and eggs pasteurized by heat or heat/ozone combination………………………………………………………143

5.4. Differential scanning calorimetry thermograms of albumin samples from untreated, heat pasteurized and combination pasteurized eggs…………………………145

5.5. SIMCA class distance projections……………………………………………147

5.6. Discriminating power for differentiation of untreated and heat pasteurized albumen……………………………………………………………………148

5.7. Discriminating power for differentiation of untreated and combination pasteurized albumen………………………………………………………………149

5.8. Discriminating power for differentiation of combination pasteurized and heat pasteurized albumen…………………………………………………………149

6.1. Internal temperature in egg albumen and yolk during heating at 57°C……….159

6.2. Thermal inactivation of *Salmonella* Enteritidis inoculated in egg yolk and shell eggs heated at at 57°C (treatment temperature) ........................................160

6.3. Inactivation of *Salmonella* Enteritidis present in yolk, albumen, or both portions of shell egg during heating at 57°C…………………………………..162

A.1. Pressure and ozone levels inside treatment chamber during treatment of shell eggs…………………………………………………………………………168

A.2. Log Reduction of *Salmonella* in whole eggs treated with heat + ozone combinations……………………………………………………………………169

B.1. Log reduction of *Salmonella* after heat treatment at 57°C for 30 or 60 minutes..176

B.2. Inactivation of *Salmonella enterica* serovars heated at 57°C…………………..177

B.3. Log reduction of *Salmonella* after treatment with UHP……………………..…178
Chapter 1
Literature review

SALMONELLA

Taxonomy

*Salmonella* is a genus belonging to the *Enterobacteriaceae* family of facultatively anaerobic Gram-negative rods commonly associated with intestinal contents. Salmonellae are most often motile and may or may not be host-adapted (LeMinor 1984). The taxonomy of the genus is somewhat unique in that it is divided into only two species; *S. enterica* and *S. bongori*. *Salmonella enterica* is further divided into six subspecies, of which *S. enterica* subsp. *enterica* (also known as subspecies I of VI) is the most notable as it comprises the vast majority of clinical isolates. Salmonellae are commonly identified as serovars, generally given the name of the geographical location of isolation (Guthrie 1992). The classification of serovars (or serotypes) is based on the antigenic formulae of isolates. This scheme, developed by Kauffman, classifies isolates based on the specific somatic (O) and flagellar (H) antigens present as well as the presence or absence of a capsular (Vi) antigen. Table 1.1 displays antigenic formulae of some of the most common clinical isolates (2006 data). The O, or somatic antigen is comprised of lipopolysaccharide associated with the cell membrane and is designated by a number. H antigens are proteins associated with flagella and may have one or two phases, the first phase designated by lower case letters and the second by numbers. Vi is the designation
for the capsular antigen, a polysaccharide which may or may not be present (LeMinor 1984). The capsular antigen is most commonly associated with *S. Typhi* and *Paratyphi* strains, the abbreviation Vi being an allusion to the altered virulence patterns of these strains when compared to the majority of other salmonellae (Guthrie 1992).

<table>
<thead>
<tr>
<th>Prevalence</th>
<th>Serovar name</th>
<th>Antigenic formula&lt;sup&gt;b&lt;/sup&gt; (O:H phase 1:H phase2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Typhimurium</td>
<td>1,4,5,12:i:1,2</td>
</tr>
<tr>
<td>2</td>
<td>Enteritidis</td>
<td>1,9,12:g,m:1,7</td>
</tr>
<tr>
<td>3</td>
<td>Newport</td>
<td>6,8:e,h:1,2</td>
</tr>
<tr>
<td>4</td>
<td>Heidelberg</td>
<td>1,4,5,12:r:1,2</td>
</tr>
<tr>
<td>5</td>
<td>Javiana</td>
<td>1,9,12:l,z:1,5</td>
</tr>
<tr>
<td>7</td>
<td>Montevideo</td>
<td>6,7:g,m,p,s:1,2,7</td>
</tr>
<tr>
<td>8</td>
<td>Muenchen</td>
<td>6,8:d:1,2</td>
</tr>
<tr>
<td>10</td>
<td>Mississippi</td>
<td>1,13,23:b:1,5</td>
</tr>
<tr>
<td>11</td>
<td>Saintpaul</td>
<td>1,4,5,12:e,h:1,2</td>
</tr>
<tr>
<td>14</td>
<td>Infantis</td>
<td>6,7:r:1,5</td>
</tr>
</tbody>
</table>

<sup>a</sup>United States Department of Health and Human Services, Centers for Disease Control and Prevention 2008  
<sup>b</sup>LeMinor 1984

**Phage typing**

Beside antigenic formula or serovar designation, another common method used to differentiate strains is phage typing. This method distinguishes bacterial cells within a
species (or serovar) on the basis of their susceptibility to specific bacteriophages. Phage typing of the salmonellae originated with *Salmonella* Paratyphi in the late 1930s and was used in epidemiological study to more accurately identify cases belonging to specific outbreaks and to pinpoint outbreak sources (Anderson et al. 1977). The method was next extended to *Salmonella* Typhimurium, due to its prevalence as an agent of foodborne disease transmission (Callow 1959). The typing of *Salmonella* Typhimurium began with the identification of twelve types, but within twenty years after the description of the method, the number of types had risen to over 200 (Anderson et al. 1977). Growth in the intricacies of the method gave rise to a complicated scheme of strain designation, assigning types with a definitive type (DT) or provisional type (PT) number (Anderson et al. 1977), though the PT abbreviation is now more commonly used to denote phage type. The utility of phage typing of *Salmonella* Typhimurium in epidemiological study facilitated its expansion to other serotypes, most notably *Salmonella* Enteritidis in the 1980s (Ward et al. 1987).

**Illness**

All members of the *Salmonella* genus (some 2400 or more serovars) are considered to be pathogenic. While some isolates, including *Salmonella* Pullorum, *Salmonella* Abortusovis and *Salmonella* Typhi (adapted to poultry, sheep and humans respectively) are known to be host-specific, many serovars are capable of causing illness in humans and animals alike. Illnesses attributed to salmonellae can be generally divided into two categories; typhoid and non-typhoid.
Enteric fever

Typhoid, or enteric fever is generally considered to be the more severe of the illnesses caused by salmonellae, but is rarer than non-typhoid illness in the developed world. The onset of enteric fever can take up to several weeks following exposure, and the infective dose is believed to be relatively high. The lowest recorded infective dose in studies conducted on human volunteers was approximately $10^5$ cells (Hornick et al. 1970). Common symptoms include fever, headache and general malaise and sometimes rash (Crump et al. 2004). This syndrome is caused exclusively by *Salmonella* Typhi and Paratyphi strains. These strains are most commonly contracted by consumption of drinking water or food that has been contaminated with fecal matter. Before effective treatment of drinking water was widespread, enteric fever was common. Typhoid fever caused more than 30 deaths per 100,000 in the United States annually in 1900, but that figure had dropped to less than 1 per 100,000 by 1950 (Armstrong et al. 1999). While rates of typhoid are low in the industrialized world, it is still a relatively common condition in less developed areas where advances in sanitation have not been as pronounced. The persistence and spread of typhoid fever is encouraged by the fact that persons who have contracted the causative pathogens may continue to shed them in their feces for years, in rare cases permanently. “Typhoid Mary,” a food handler thought to be responsible for causing several typhoid outbreaks in the United States in the early 1900s is an example of this phenomenon (Guthrie 1992). While typhoid fever can be treated with antibiotics, resistant strains of *Salmonella* Typhi are becoming more common, greatly complicating the treatment of this condition (Rowe et al. 1997).
Gastroenteritis

Gastroenteritis is the more common of the illnesses resulting from contact with salmonellae and is generally transmitted by consumption of contaminated food. This illness is commonly known as salmonellosis or enterocolitis, and is characterized by nausea, diarrhea and fever. Symptoms generally cease in less than one week in adults and illness is often mild enough that medical treatment is not sought. This leads to what is believed to be a great underrepresentation of the actual prevalence of salmonellosis due to underreporting (Hope et al. 2002, Schroeder et al. 2005). The Centers for Disease Control and Prevention (CDC) estimates that there are approximately 1.4 million cases of salmonellosis in the United States each year, (Mead et al. 2000), and it is currently the most common notifiable foodborne illness in this country.

Sequelae

Though Salmonella gastroenteritis is generally resolved without complication, it occasionally precedes other more serious symptoms in some individuals. The most common sequela of salmonellosis is reactive arthritis. This syndrome results from an invasive infection which causes an immune-mediated inflammatory response to Salmonella O antigens (Maki-Lkola and Granford 1992). Onset of reactive arthritis is typically several weeks after gastrointestinal symptoms subside. Some authors have noted a correlation between the length and severity of gastroenteritis and the presentation and severity of subsequent arthritis symptoms (Maki-Lkola and Granford 1992, Dworkin et al. 2001). One study estimated the prevalence of reactive arthritis following salmonellosis at as high as 29% (Dworkin et al. 2001), though rates are typically believed
to be somewhat lower. The condition most often resolves within several months, but may be chronic in some patients (Maki-Lkola and Granford 1992). The main risk factor for development of post-Salmonella reactive arthritis is the presence of the HLA-b27 surface antigen. When it is present, severity and duration of symptoms are generally increased (Leirisalo et al. 1982). Other sequelae are rare in developed countries, but Salmonella is a known cause of both meningitis and osteomyelitis in children of the developing world (Lee et al. 1999, Huang et al. 1997, Adeyokunnu and Hendrickse 1980).

**Salmonella in food**

A recent outbreak (2009) involving peanut products has highlighted the emerging challenge of controlling salmonellae in different food environments. In a study conducted by Alford and Palumbo (1969), several strains of *Salmonella* were able to grow in ground pork with salt concentrations of up to 5% at temperatures as low as 10°C. In peanut butter, high fat content and low water activity combine to increase the heat resistance of bacterial cells. In a 2006 study, heat treatments as severe as 90°C for 50 minutes resulted in only 3.6 log decrease of *Salmonella* (Shachar and Yaron 2006). The combination of high fat and low water activity was purported to increase survival of *Salmonella* in chocolate during storage; viable cells were recovered as long as nine months after inoculation (Tamminga et al. 1976). It has also been suggested that these properties may protect cells from the acidic environment of the stomach following consumption, effectively lowering the infective dose. In a 1982 outbreak of *Salmonella* Napoli attributed to chocolate bars, infectious dose was estimated to be as few as 10-40 cells (Greenwood and Hooper 1983).
Outbreak vehicles for *Salmonella* are diverse (Table 1.2), however, the foods most commonly associated with *Salmonella* contamination have historically been poultry and eggs. The largest recorded outbreak of *Salmonella enterica* serovar Enteritidis (*Salmonella Enteritidis*) was caused by contaminated ice cream in 1994. Pre-pasteurized ice cream mix was carried in a tanker that had previously carried raw eggs and was not properly sanitized prior to product change-over. The resulting cross-contamination led to over 200,000 illnesses in several states (Hennessy et al. 1996). It is estimated that 1 of every 20,000 eggs produced in the United States is internally contaminated with *Salmonella* Enteritidis (Ebel and Schlosser 2000), and external contamination may be significantly more prevalent (Musgrove et al. 2005b). Consumption of eggs is the number one risk factor associated with *Salmonella* Enteritidis infection (Hope et al. 2002). Egg contamination is a problem that was given increasing attention during the 1980s due to the sharp rise in isolation of *Salmonella* Enteritidis from ill patients; between 1976 and 1995, the percentage of total clinical *Salmonella* isolates belonging to the Enteritidis serovar rose from approximately 5% to greater than 25% (Hogue et al. 1997, Humphrey et al. 1988). Contaminated eggs have been estimated to result in 180,000 to over 660,000 illnesses each year (Hope et al. 2002, Schroeder et al. 2005), the cost of which is approximated at $150-870 million each year (Hope et al. 2002), due mainly to health care costs and productivity loss. Risk of contracting illness from contaminated eggs is increased by a number of practices including the storage of eggs at room temperature, undercooking and pooling large numbers of eggs, common procedures in restaurants, catering operations and institutional settings. As a result of unsafe
handling and undercooking, risk of *Salmonella* Enteritidis illness is increased by restaurant dining (Passaro et al. 1996).

### Table 1.2 Examples of recent *Salmonella* outbreaks

<table>
<thead>
<tr>
<th>Year</th>
<th>Serovar</th>
<th>Confirmed cases</th>
<th>Suspected food vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>Typhimurium</td>
<td>183</td>
<td>Fresh tomatoes</td>
</tr>
<tr>
<td>2007</td>
<td>Wadsworth</td>
<td>65</td>
<td>Veggie Booty (extruded snack)</td>
</tr>
<tr>
<td>2007</td>
<td>Tennessee</td>
<td>425</td>
<td>Peanut butter</td>
</tr>
<tr>
<td>2008</td>
<td>Agona</td>
<td>28</td>
<td>Ready-to-eat breakfast cereal</td>
</tr>
<tr>
<td>2008</td>
<td>Litchfield</td>
<td>51</td>
<td>Cantaloupe</td>
</tr>
<tr>
<td>2009</td>
<td>Saintpaul</td>
<td>1442</td>
<td>Jalapeno/Serrano peppers</td>
</tr>
<tr>
<td>2009</td>
<td>Montevideo</td>
<td>202</td>
<td>Salami</td>
</tr>
</tbody>
</table>

*a*United States Department of Health and Human Services, Centers for Disease Control and Prevention 2009.

## EGGS

### Egg laying and structure

The process of egg laying, illustrated in Figure 1.1 begins in the hen ovary. Though hens possess two ovaries, in the vast majority, only the left is functional (Bell 2002). Within the ovary, the germ cell is surrounded by several alternating layers of white and yellow, also called light and dark yolk. The yolk material is produced in the liver and transported to the ovary by the circulatory system (Bell 2002). The fully-formed yolk then passes into a long tube-like structure called the oviduct. The oviduct can be generally broken down into sections by the portion of the finished egg that is secreted. These are the infundibulum, isthmus and uterus where, in succession, the yolk enters the oviduct and is surrounded by the albumen, followed by inner and outer shell membranes, and the egg shell. The fully-formed egg then enters the vagina and is
expelled through the cloaca (Romanoff and Romanoff 1949). Several factors can influence the production of an individual hen, including breed, age, exposure to light and temperature (American Egg Board 2008).

Figure 1.1 Hen reproductive system
Modified from (Romanoff and Romanoff 1949)

Avian eggs have three main components; the shell, the albumen and the yolk (Figure 1.2). The shell accounts for approximately 10% of the total weight of an egg and the bulk of the shell material is comprised of calcium carbonate, though it also contains a
small amount of protein (Romanoff and Romanoff 1949). Numerous small pores (10-65 μm in diameter) run through the shell, allowing gas exchange (Kobayashi et al. 1997); (Board and Tullett 1977). As technology has improved, more and more layers of eggshell have been identified. The outermost layer of the shell is the cuticle, followed by the vertical crystal layer, palisade, mammillary layer and shell membranes. The cuticle is a fine meshwork consisting mainly of collagen that covers the entire outer surface of the egg and is itself comprised of two distinct layers (Fraser et al. 1999). It is thought to be the first defense against penetration by bacteria, but is often removed by conventional washing procedures. The vertical crystal layer is a thin section of calcite crystals formed around an organic matrix aligned vertically relative to the shell. Following the vertical crystal layer is the palisade, which is the thickest layer in the eggshell. The organic matrix in this layer is aligned perpendicular to the vertical crystal layer (Fraser et al. 1999). The mammillary layer terminates in a series of conical protrusions that come into direct contact with the first of the two shell membranes. On the interior of the shell surface there are two gas permeable membranes, the outer and inner shell membranes, onto which the shell material is deposited as the shell forms. Both are largely proteinaceous in nature and resemble net-like structures, with both the diameter of individual fibers and overall thickness of the outer membrane being greater than that of the inner membrane (Bellairs and Boyd 1969). The two shell membranes are not connected and separate from each other at the blunt end of the egg where the air sac is formed; the outer membrane remains in contact with the shell while the inner membrane encloses the albumen (Bellairs and Boyd 1969, Romanoff and Romanoff 1949). It has
been proposed that the two membranes are themselves also composed of several layers of varying thicknesses (Tan et al. 1992).

The albumen comprises the majority of the egg’s weight (approximately 60%) and is composed mainly of water (88% of total albumen weight) (Zeidler 2002b) and proteins (over 85% of albumen solids), including ovalbumin, ovomucoid, ovomucin and several others (Table 1.3) (Romanoff and Romanoff 1949). The albumen is arranged in two distinct phases (with two layers of each); thin and thick albumen. The only notable difference between the two phases is a higher concentration of ovomucin in the thick portion. The viscosity of egg albumen is attributed specifically to this protein, which
forms a sort of tubular scaffolding that holds the albumen together. The yolk is anchored in the egg by protein fibers called the chalazae, which together with a narrow layer of thick albumen, constitute the innermost layer of albumen, the chalaziferous layer. This layer is surrounded by a limited amount of thin albumen. The third layer comprises the bulk of the albumen. Generically termed the “thick albumen,” the albuminous sack is where the fibers of the chalazae end. Surrounding this is another, more abundant layer of thin albumen (Romanoff and Romanoff 1949).

Table 1.3 Major albumen protein characteristics

<table>
<thead>
<tr>
<th>Protein</th>
<th>% Albumen dry weight</th>
<th>Isoelectric point</th>
<th>Denaturation temperature (°C)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>54</td>
<td>4.5</td>
<td>84</td>
<td>Storage</td>
</tr>
<tr>
<td>Ovotransferrin</td>
<td>12</td>
<td>6.1</td>
<td>61</td>
<td>Iron binding protein</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>11</td>
<td>4.1</td>
<td>70</td>
<td>Trypsin inhibitor</td>
</tr>
<tr>
<td>Ovomucin</td>
<td>3.5</td>
<td>4.75</td>
<td>N/A</td>
<td>Structural</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>3.4</td>
<td>10.7</td>
<td>75</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>Globulins (G2 and G3)</td>
<td>8</td>
<td>4.8-5.5</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

(Powrie and Nakai 1986, Stevens 1991)

The innermost portion, the yolk, contains the vast majority of the lipids in the egg and from a nutritional standpoint, the bulk of the egg’s calories. More than half of the dry weight of egg yolk (60%) is comprised of triglycerides (Bell 2002). Oleic acid is the most abundant fatty acid contained in yolk lipids, accounting for approximately 40% of the total, while saturated fatty acids (palmitic and stearic acids) account for approximately 35% of yolk lipids, and the remainder contains a mixture of other unsaturated fatty acids (Privett et al. 1962). Another notable constituent of egg yolk is
lecithin, an amphipathic substance used commercially as an emulsifier and the basis of the use of raw egg yolk in many home cooking applications. Alternating layers of yellow and white yolk surround the central latebra, a small sphere connected to a channel through the yolk, which is itself composed of white yolk. The neck of latebra, the channel extending through the yolk, terminates at the blastodisc, which would, if fertilized, become an embryo. Surrounding the final thin layer of white yolk is the vitelline membrane, a netlike membrane composed primarily of collagen that keeps the yolk contents separated from the albumen (Romanoff and Romanoff 1949).

**Egg allergy**

The proteins of the albumen are responsible for the allergenicity of egg. As one of the most common food allergens, any food containing egg-based ingredients must state their presence unambiguously on its label. Egg allergy is particularly prevalent in children, affecting an estimated 1-2% (Eggesbo et al. 2001). The majority of sufferers (up to 91%) will outgrow the allergy by age 18 (Eggesbo et al. 2001). Any of the egg white proteins may be responsible for allergic reaction in a certain individual. Research has demonstrated that heating may alleviate reactions in some individuals, indicating specific allergy to the more heat labile egg proteins (i.e. avidin), while those affected by heated egg white show no reaction to ovomucin-depleted albumen (Urisu et al. 1997).

**Egg market**

Just over 90.2 billion eggs (all types) were produced in the United States in 2009 by approximately 340 million laying hens. Of that number, approximately 77.5 billion
were table (shell) eggs. Iowa is currently the number one egg producing state in the country, followed by Ohio and Pennsylvania (United States Department of Agriculture, National Agricultural Statistics Service, Agricultural Statistics Board 2010). Eggs are eaten in over 95% of American households and the average American eats over 250 eggs per year (American Egg Board 2003).

**Egg processing**

In USDA approved facilities, eggs are washed and graded before packaging. Table 1.4 displays criteria used by inspectors to determine egg grade. Eggs are also categorized roughly by size, with categories based on the combined weight of one dozen eggs. Size category information is summarized in Table 1.5. After washing, egg shells may be coated with oil, but this is an optional procedure. The process of washing, rinsing and drying is required to be continuous. Wash water must be kept at no less than 90°F (32.2°C) and must also be at least 20°F (6.7°C) warmer than the internal temperature of unwashed eggs. Water is replaced a minimum of once every four hours, more often as needed. Wash water generally contains an approved sanitizer, such as chlorine, and often an antifoaming agent to promote re-use. Eggs must be dry before packaging. Once packaged, regulations require all eggs to be held at no less than 45°F (7.2°C) in a humid environment to prevent weight loss (United States Department of Agriculture 2008). Research as demonstrated that as regulated, washing can provide significant decreases in external microbial count (Hutchison et al. 2004, Musgrove et al. 2005b). However, if parameters, including water temperature and pH and chlorine concentration, are not
strictly controlled, washing may promote cross contamination and/or penetration of bacteria through the shell to the egg contents (Bartlett et al. 1993, Hutchison et al. 2004).

Table 1.4 USDA quality standards for egg grading

<table>
<thead>
<tr>
<th>Grade</th>
<th>Shell</th>
<th>Air cell</th>
<th>Albumen</th>
<th>Yolk</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Clean or slightly stained. Unbroken, abnormal</td>
<td>Depth &gt;3/16 inch.</td>
<td>Weak, watery, blood/meat spots present.</td>
<td>Enlarged and flattened.</td>
</tr>
<tr>
<td>Dirty</td>
<td>Prominent stains. Unbroken</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Check</td>
<td>Broken or cracked, not leaking</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*aModified from United States Department of Agriculture, Agricultural Marketing Service.

Table 1.5 Recognized egg weight classes

<table>
<thead>
<tr>
<th>Class</th>
<th>Minimum weight per dozen (oz)</th>
<th>Average weight per egg (oz/g)</th>
<th>Minimum weight per egg (oz/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jumbo</td>
<td>30</td>
<td>2.5</td>
<td>2.42</td>
</tr>
<tr>
<td>Extra large</td>
<td>27</td>
<td>2.25</td>
<td>2.17</td>
</tr>
<tr>
<td>Large</td>
<td>24</td>
<td>2</td>
<td>1.92</td>
</tr>
<tr>
<td>Medium</td>
<td>21</td>
<td>1.75</td>
<td>1.67</td>
</tr>
<tr>
<td>Small</td>
<td>18</td>
<td>1.5</td>
<td>1.42</td>
</tr>
<tr>
<td>Peewee</td>
<td>15</td>
<td>≤ 1.25</td>
<td>none</td>
</tr>
</tbody>
</table>

*aModified from Zeidler 2002a.
Antimicrobial features of egg

Hen eggs possess a number of natural obstacles to bacterial contamination. The first of these is the cuticle that encircles the egg shell. While the cuticle does not obstruct gas exchange, it is thought to present a barrier that discourages bacterial cells from entering the pores of the egg shell. It was originally believed that the only other defense offered by the shell was the physical barrier of the shell membranes and that the net-like structure of the membranes was able to trap some cells and prevent them from entering the albumen. This theory was examined by Lifshitz and colleagues, who found that the inner shell membrane was the most important eggshell component for the physical prevention of internal contamination (Lifshitz et al. 1964). One study involved the incubation of several bacterial species with an eggshell membrane extract. Incubation in 9% extract (vol/vol) resulted in a reduction of Salmonella Enteritidis of greater than 40%. When treated cells were subsequently heated, the $D$-value of these cells was decreased by over 80% compared to cells that had not been subjected to membrane extract (Poland and Sheldon 2001). More recently, several antimicrobial compounds have been isolated from egg shells and eggshell membranes. Mine and colleagues (2003) demonstrated that when incubated with soluble proteins isolated from the eggshell matrix, some Gram positive organisms were inhibited. Gram negative species, including Salmonella were found to be much less susceptible to observed membrane damage in this study. The authors did not identify specific proteins that might be responsible for observed effects. Shell membranes contain a number of enzymes that act as antimicrobials, including lysozyme and β-N-acetylglucosaminidase. The combination of these enzymes poses a threat to the stability of the bacterial cell membrane (Ahlborn and Sheldon 2005).
Albumen is also known to contain a number of antimicrobial agents. The most well known of these is lysozyme, a protein with enzymatic activity capable of breaking glycosydic linkages in peptidoglycan, leading to membrane damage and lysis. Iron restricting proteins, most notably ovotransferrin, have also been identified. Ovotransferrin molecules bind iron III, making it unavailable for use by bacterial cells. This mechanism is thought to be the egg’s main defense system against internal bacterial growth. When *Salmonella* Enteritidis was inoculated into albumen, the addition of supplementary iron enhanced the survival of the pathogen (Kang et al. 2006), indicating that iron limiting conditions may contribute to the bacteriostatic activity of egg albumen. In addition to the presence of antimicrobial proteins, the pH of egg albumen is relatively high, ranging from 8.1 to 9.7 depending on the age of the egg (Romanoff and Romanoff 1949), well outside the optimal range for many organisms, including *Salmonella* spp. These factors combine to produce a hostile environment for invading microbes. If however, bacterial cells are able to reach the yolk, growth occurs readily, as will be discussed later.

**Egg contamination**

Despite the barriers previously mentioned, egg contamination is not uncommon and a number of bacterial species have displayed the ability to survive or even replicate within eggs. *Pseudomonas* spp. have been known to cause egg spoilage (Board and Tranter 1986). A number of fungal genera, including *Aspergillus*, *Penicillum* and *Mucor* have also been isolated from the interior of egg shells or egg contents (Ayres 1960); (Romanoff and Romanoff 1949). While these organisms can lead to product loss through
spoilage, egg spoilage is not particularly common. The issue of preeminent concern in egg sanitation is the contamination of eggs with *Salmonella* Enteritidis, which causes a significant number of illnesses annually in the United States. *Salmonella* may be introduced into the laying environment in several ways, including presence of rodents or insects and use of contaminated feed. It has been shown that once introduced, *Salmonella* Enteritidis can persist for longer than 26 weeks (Davies and Breslin 2003b).

**Horizontal contamination of eggs with *Salmonella* Enteritidis**

Horizontal contamination refers to the acquisition of contaminants, often originating from feces or soil, after laying. These contaminants may be present on the shell of the egg and may subsequently be internalized. There are several factors that can contribute to the increased likelihood of this scenario. Firstly, when the egg exits the cloaca, its internal temperature is the same as the body of a hen, 42°C (Romanoff and Romanoff 1949). The rapid cooling of the egg after laying and especially upon prompt refrigeration can lead to the generation of negative pressure in the egg. Some bacteria, including *Salmonella* Enteritidis can be internalized with air or moisture that is pulled into the egg as a result of this pressure differential (Fajardo et al. 1995, Haines and Moran 1940, Romanoff and Romanoff 1949). Schoeni and colleagues reported that prompt refrigeration of warm eggs (35°C) enhanced the internalization of three *Salmonella* serovars (Schoeni et al. 1995). Any damage to the integrity of the shell, even hairline cracks, greatly increases the risk of internal contamination. Egg washing, as previously described, can also promote horizontal contamination through the removal of the cuticle from the shell exterior. Removal of the cuticle results in the exposure of the shell pores,
making penetration more likely. Washing can also serve as a conduit of cross contamination (Musgrove et al. 2005b). Any other shell defects or cracks due to handling or rapid cooling also increase the risk of horizontal contamination (Fajardo et al. 1995). While egg processing may play a role in penetration of egg shells by bacteria, it has been reported that neither the breed of hen nor the type of cage used to house hens contributes significantly (Messens et al. 2007). Horizontal contamination was originally thought to be the main method of introduction of *Salmonella* Enteritidis into eggs, but it is now widely believed that vertical contamination is more common.

**Vertical contamination of eggs with *Salmonella Enteritidis***

Vertical contamination of eggs, also known as transovarian transmission, refers to the deposition of pathogen in the forming egg by transfer from the reproductive tissues of an infected hen. In this scenario, an asymptomatic, systemic infection in the laying hen leads to the presence of *Salmonella* Enteritidis cells throughout the reproductive tract. Numerous studies have shown that hens may be infected with *Salmonella* spp. in a number of ways, including orally, (Gast et al. 2007), intravenously, (Okamura et al. 2001a), or intravaginally (Gast et al. 2007, Okamura et al. 2001b). Because both the ovary and the oviduct may be contaminated, cells may be shed at any point in the development of the egg and may be found after laying in either the albumen or yolk (Gast et al. 2007, Okamura et al. 2001a, Gast and Holt 2000a, Humphrey et al. 1991, Humphrey et al. 1989). However, contamination is believed to occur most commonly at the site of the vitelline membrane due to the fact that cells are found more often associated with the albumen than yolk (Humphrey et al. 1991, Thiagarajan et al. 1994).
Vertical transmission of *Salmonella* seems to occur only sporadically even in heavily infected hens. In a study of eggs laid by naturally infected hens, 1119 eggs were tested and *Salmonella* Enteritidis was recovered from only 11 of these samples (Humphrey et al. 1989). Another study of fifteen flocks known to be naturally contaminated found the rate of vertical contamination to be only 0.6% (Humphrey et al. 1991). In studies using artificially inoculated hens, (doses of approximately $10^6 – 10^9$ colony forming units), rates of internal contamination ranged from 0 – 7.5%, with levels of external contamination at up to 27.5% (Okamura et al. 2001a, Okamura et al. 2001b, Gast and Holt 2000a, Gast et al. 2004). Naturally contaminated eggs also appear to contain very low internal levels of *Salmonella*, averaging approximately ten cells per egg (Humphrey et al. 1991). There does exist, however, the possibility that cells deposited near the yolk may penetrate the vitelline membrane and grow to very high levels. This happens infrequently, but storage temperature plays a major role in both penetration and growth (Gast and Holt 2000b, Gast et al. 2006). The issue of vertical contamination is particularly troublesome from a food safety standpoint, because traditional processing (i.e. washing and application of sanitizers) can do nothing to enhance safety of products contaminated by this route.

**Specific advantages of *Salmonella* Enteritidis in hen tissue colonization and transmission to eggs**

It has often been noted that though eggs are exposed to several serovars of *Salmonella*, only *Salmonella* Enteritidis is highly associated with egg products. A wealth of research has been performed to assess the ability of *Salmonella* Enteritidis to
contaminate eggs relative to other salmonellae. In one *in vitro* study, *Salmonella* Enteritidis was found to adhere to egg follicles in greater numbers than *Salmonella* Typhimurium and to vaginal epithelial cells in greater numbers than serovars including Typhimurium, Agona and Heidelberg (Mizumoto et al. 2005). The authors suggested that the type of lipopolysaccharide produced by a given serovar may play a role in its interaction. Another study found the frequency of colonization of the reproductive tract to be similar between *Salmonella* Enteritidis and *Salmonella* Typhimurium, but could only isolate *Salmonella* Enteritidis from forming eggs (Keller et al. 1997). In experiments conducted in 2001, Okamura and colleagues inoculated mature hens intravenously with six different *Salmonella* serovars; while all were able to colonize reproductive tracts, *Salmonella* Enteritidis was found to do so at significantly higher levels and was again the only serovar recovered from laid eggs (Okamura et al. 2001a). In another study conducted by the same group, hens were inoculated intravaginally. Similar results were observed with regard to reproductive tissues, and all serovars were found associated with egg shells but only *Salmonella* Enteritidis and *Salmonella* Typhimurium were isolated from egg contents (Okamura et al. 2001b). In more recent studies by Gast and colleagues, hens were inoculated orally with either *Salmonella* Enteritidis or *Salmonella* Heidelberg with no resulting differences in tissue colonization between serovars. Both serovars were recovered from egg contents, but again *Salmonella* Enteritidis was found at a higher frequency (Gast et al. 2007, Gast et al. 2004). All of this research is in accordance with the real-world observations that *Salmonella* Enteritidis is the serovar most often found in association with eggs and egg containing foods, and illness from *Salmonella* Enteritidis is associated with eggs and egg containing foods more often than
any other vehicle. This supports the theory that there is some fundamental difference between *Salmonella* Enteritidis and other *Salmonella* serovars which allows it excel not only in colonization of hen reproductive tissues, but also in transmission to forming eggs and survival in eggs during storage.

In 2003, Lu and associates identified the *yafD* gene in *Salmonella* Enteritidis. When transformed into *Salmonella* Typhimurium, the transformant displayed significantly increased survival when inoculated into albumen and incubated at 37°C. Likewise, *Salmonella* Enteritidis *yafD* deletion mutants displayed significantly decreased survival under the same conditions (Lu et al. 2003). The authors noted a homology between YafD and members of the endonuclease-exonuclease-phosphatase family, which led them to theorize that the mechanism of protection afforded by the *yafD* gene product is related to DNA repair. To test this hypothesis, wild type and deletion mutants were treated with hydrogen peroxide and UV radiation. Results showed significantly increased survival in wild type cells. To verify the applicability of this mechanism in eggs, a nuclease assay was performed, confirming both endonuclease and exonuclease activity of egg albumen (Lu et al. 2003).

A 2006 study by the same group used transposon mutagenesis to identify additional genes important to *Salmonella* Enteritidis survival in albumen. Authors identified several mutants with increased susceptibility to albumen, the majority of which carried insertions in sequences related to cell wall structure and function or amino acid metabolism. One sequence absent from *Salmonella* Typhimurium was also identified and confirmed to play a role in survival. From this work, it was concluded that cell wall integrity is a main contributor to bacterial survival in albumen and that *Salmonella*
Enteritidis possess certain genetic variations that make it more suited than other serovars to survival in the environment of the egg (Clavijo et al. 2006). In 2008, Gantois et al. published a study citing the importance of $rfbH$, a gene involved in the synthesis of O antigens (Gantois et al. 2009). The authors observed increased transcription of this gene in cells incubated in albumen at room temperature. A knockout mutant was unable to grow under identical conditions, and was unable to survive in albumen at higher temperatures. A mechanism to explain these observations was not proposed, but the findings support the suggestion of Mizumoto et al. that altered *Salmonella* Enteritidis lipopolysaccharide may play a role in egg contamination.

**Phage type 4**

As a result of increasing prevalence of *Salmonella* Enteritidis-related outbreaks, a phage typing system was developed for this serovar in the 1980s using ten phages and identifying a total of 27 distinct types (Ward et al. 1987). In the United States, the most commonly observed phage types are 8 and 13a. Phage type 4 (PT4) is known to be extremely prevalent in Europe, most especially in the United Kingdom. The first known outbreak caused by PT4 in the United States occurred in a Texas restaurant in 1993 (Boyce et al. 1996). Because the spread of PT4 geographically has been observed, it has been theorized that outbreak strains are descendent of a common ancestral strain possessing unique characteristics. Several molecular typing methods, including genomic DNA fingerprinting, ribotyping and enzyme typing, have been applied to PT4 isolates. Results show surprisingly little variation, a fact that supports the common ancestor theory (Helmuth and Schroeter 1994). It has been reported that PT4 isolates demonstrate
increased virulence in chicks when compared to other phage types (Gast and Benson 1995) and that some possess the ability to invade the muscle tissue of poultry (Humphrey et al. 1988).

**Survival and growth of *Salmonella* in eggs**

As previously discussed, the egg is not a hospitable environment for bacterial cells. However, there are three major factors contributing to the likelihood of survival; i. site of contamination (albumen vs. yolk), ii. inoculum level, (applicable mainly to artificially contaminated eggs) and iii. storage temperature. Due to reasons discussed earlier, the yolk is a much more hospitable environment for bacterial growth than the albumen. Fleischman and colleagues inoculated eggs with 2-3 log CFU *Salmonella* Enteritidis and incubated them at either 4, 8, 15, 27 or 37°C for up to 48 hours. The researchers found that cells inoculated in albumen did not grow at any temperature, while cells inoculated into yolk grew at all but the two lowest temperatures. Cells placed on the vitelline membrane grew only at the two highest temperatures, and did not penetrate into the yolk contents at any temperature (Fleischman et al. 2003). In a Japanese study, 10³ CFU were placed on the vitelline membrane and eggs were stored at 25°C. *Salmonella* Enteritidis was recovered from yolk contents of some samples after three days of storage; after an additional three days, 40% of yolks tested positive, suggesting that diffusion through the yolk membrane is time dependant (Murase et al. 2006). In an earlier study, wherein eggs were inoculated with slightly higher levels of cells (10⁴ CFU) in the albumen and stored at 2-8 or 26°C, penetration and rapid growth in the yolk was observed after two days of incubation at the higher temperature. Penetration was not
detected at the lower temperature after up to 16 days of storage, illustrating the importance of storage temperature on internal levels and migration of *Salmonella* Enteritidis within eggs (Hammack et al. 1993). An *in vitro* study by Gast and associates supports this finding. The authors report that refrigeration immediately after inoculation of 100 CFU onto the surface of egg yolk (separated from whole egg) prevented penetration into yolk contents for at least 24 hours. In samples prepared similarly but stored at 30°C, penetration was observed after only two hours and 40% of samples tested positive after 24 hours of storage (Gast et al. 2006). In a comprehensive set of experiments conducted by Chen and associates, eggs were inoculated in the albumen with $10^2$, $10^4$, or $10^6$ CFU and stored at 4, 10, or 22°C for up to six weeks. Other parameters were measured concurrently, most notably the force required to rupture the vitelline membrane. At the lowest and middle levels of inoculation, populations decreased or stayed static over the storage period regardless of storage temperature. However, at higher levels, population increased even at 4°C. The investigators found that the strength of the membrane was maintained better at lower temperatures and theorized that this played a part in the more rapid growth seen at 22 and 10°C (Chen et al. 2005). This inference is supported by studies that report enhanced survival of *Salmonella* Enteritidis inoculated in eggs that have been stored, leading to weakening of the vitelline membrane, consequently making it easier for cells to pass through or allowing nutrients to leach out into the surrounding albumen (Humphrey and Whitehead 1993). Because vertical contamination most often results in the deposition of very few cells into the albumen of the egg, results from the cited studies seem to indicate that storage temperature, or avoidance of temperature abuse is the most important influence on egg safety. This
highlights the need for strict regulations for producers and sellers as well as consumer education regarding safe egg handling.

**Egg safety initiatives**

The number of illnesses from *Salmonella* Enteritidis in the United States rose steadily through the 1980s until 1996 (Olsen et al. 2001). During this time, *Salmonella* Enteritidis also overtook *Salmonella* Typhimurium, though only briefly, as the serovar most commonly isolated from humans (Olsen et al. 2001). In 1990, the USDA issued requirements that breeding flocks be monitored and kept *Salmonella* Enteritidis free. Chicks were to be tested and if positive results were obtained, the flock was euthanized (Hogue et al. 1997). Additionally, a traceback program was initiated by the USDA. The aim of this program was to identify the flocks from which outbreak-causing eggs had originated. Once identified, hens were tested and if confirmed positive, their eggs were diverted to be sold as pasteurized egg product (Mason 1994); this program is still in operation, but is now controlled by the FDA (Hogue et al. 1997). When these initiatives failed to produce a reduction in *Salmonella* Enteritidis illness, a pilot quality control program was launched in Pennsylvania in 1992.

The *Salmonella* Enteritidis Pilot Project (SEPP) required testing of flocks and laying environment for presence of *Salmonella* Enteritidis, more effective cleaning of laying environments and better pest control plans (Hogue et al. 1997). If *Salmonella* Enteritidis was found, eggs themselves were screened for presence of the pathogen. Its identification required eggs to be diverted to pasteurization (Hogue et al. 1997). During the Pilot Project, no outbreaks were traced back to participating farms (Mason 1994).
Due to the success of the program, it was continued under control of the state as the Pennsylvania Egg Quality Assurance Program. Several other quality assurance programs are now in effect at the state level.

The trend of increasing egg-associated illness observed in the United States was mirrored throughout Europe, which caused significant concern regarding egg safety. In Great Britain, where levels of *Salmonella Enteritidis* in eggs were uncommonly high, the British Egg Industry Council introduced the “Lion Quality” designation in 1998 in response to drastically reduced demand for eggs. This optional designation required producers to institute a number of safety measures applying to breeding stock, layers and packaged eggs. A summary of the requirements for qualification is given in Table 1.6. Currently, 85% of shell eggs produced in Great Britain bear the lion quality stamp (British Egg Industry Council 2007). The implementation of the procedures required to earn the Lion stamp has led to significant decreases in egg-associated illness (Gillespie 2004).
Table 1.6 Summary of requirements for "Lion Quality" eggs (Great Britain)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Production step</th>
<th>Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breeding flocks</td>
<td>Flocks and hatcheries must be tested for <em>Salmonella</em>, birds testing positive are slaughtered.</td>
</tr>
<tr>
<td>Laying hens</td>
<td>All laying hens must be vaccinated against <em>S. Enteritidis</em> and must be tested for <em>Salmonella</em> before transportation to farm</td>
</tr>
<tr>
<td>Hen feed</td>
<td>All feed must be free of ingredients derived from mammalian or avian sources and a number of other materials.</td>
</tr>
<tr>
<td>Laying environment</td>
<td>Regular swabbing for <em>Salmonella</em> is required, farms must have a policy in place to deal with all waste products in a way that minimizes environmental impact on the community.</td>
</tr>
<tr>
<td>Animal welfare</td>
<td>Induced molting is banned, eggs produced by caged hens may not be labeled as “farm eggs” or display pastoral imagery.</td>
</tr>
<tr>
<td>On-farm egg handling</td>
<td>All eggs must be stored below 20°C and must be packed within three days of lay.</td>
</tr>
<tr>
<td>Packing center</td>
<td>HACCP plan and rodent control procedures required.</td>
</tr>
<tr>
<td>Egg labeling and packing</td>
<td>Egg shells and packages require “best before” date a maximum of 27 days post-lay. Eggs carry Lion Quality mark for identification, producer identification code and mode of production (organic, free-range, barn, cage) must be stamped on shell.</td>
</tr>
<tr>
<td>Auditing</td>
<td>Inspection by independent agency is required, self-audit to be performed at least every six months.</td>
</tr>
</tbody>
</table>

\textsuperscript{a}British Egg Industry Council 2007
As a response to the severity of *Salmonella* Enteritidis outbreaks in the United States, the President’s Council on Food Safety released a plan entitled “Egg safety from production to consumption: an action plan to eliminate *Salmonella* Enteritidis illnesses due to eggs.” in December of 1999. The plan advised more rigorous sanitation in laying environments as well as increased testing of laying flocks and the diversion of eggs from *Salmonella* Enteritidis positive flocks to liquid egg products, which require pasteurization. It also advocated the implementation of new technology to produce pasteurized whole shell eggs (President's Council on Food Safety 1999). The plan put forth a goal of 50% reduction in egg-associated illness by 2005, this goal was not met successfully. Egg safety is also addressed in the Healthy People 2010 report, which also aims for 50% reduction (from 1997 levels; a target of 22 annual outbreaks or 6.8 cases per 100,000 people) by 2010 (United States Department of Health and Human Services 2000).

**Regulations**

In December 2000, the US Food and Drug Administration (FDA) published a final rule requiring a safe handling statement to appear on all eggs which have not been subjected to pasteurization. This rule took effect on September 4, 2001 and requires the following statement to appear on the upper portion of the packaging in type measuring at least one-sixteenth inch: “**SAFE HANDLING INSTRUCTIONS:** To prevent illness from bacteria: keep eggs refrigerated, cook eggs until yolks are firm, and cook foods containing eggs thoroughly” (United States Food and Drug Administration 2000a).

Another final rule has just recently been passed (July 2009). This rule, which takes effect 12-36 months after its announcement (depending on the size of the establishment), outlines several steps that will be required to increase the safety of shell
eggs. These include the establishment of pest control and other plans to prevent contamination of the laying environment as well as monitoring laying environment for *Salmonella* Enteritidis. If tests are positive, the laying house must be decontaminated and eggs from the affected flock are tested over an eight week period for contamination. If any eggs test positive, all eggs from the affected flock will be diverted to pasteurized applications for the duration of the laying period. The rule will also require that all eggs be stored at 45°F (or lower) no more than 36 hours after laying (United States Food and Drug Administration 2009).

**Treatments for enhanced egg safety**

**Egg surface**

The presence of *Salmonella* on the surface of eggs is not uncommon. Musgrove and co-workers (2005a) isolated *Salmonella* from over 8% of washed eggs tested. Due to the threat of horizontal contamination of eggs with *Salmonella*, many researchers have focused on ways to remove these organisms from egg shells without introducing detrimental effects to the egg components. In 1966, researchers at Iowa State University investigated the use of several commercial disinfectants to reduce *Salmonella* inoculated onto egg surface. While all sanitizers were effective at reducing surface populations, none were capable of inactivating cells that had reached the shell membranes (Rizk et al. 1966).

In 2003, Davies and Breslin used a number of non-traditional methods to reduce *Salmonella* on egg shells. The authors found no increased reduction in contamination from treatment with ionized air, dry ozone gas, moist ozone gas or an herbal sanitizer.
when compared to washing in distilled water (Davies and Breslin 2003a). A 2004 study investigated the use of thermoultrasonication, finding that the combination of heat (54°C for 5 min) and ultrasound resulted synergistic inactivation of *Salmonella* Enteritidis (Cabeza et al. 2004). Further studies on the quality of eggs treated with this process demonstrated that changes in egg quality were not detectable (Cabeza et al. 2005). In research conducted by Rodriguez-Romo and Yousef, (2005), combinations of ozone gas and UV radiation were used to sanitize egg shells. This study also demonstrated synergistic inactivation of *Salmonella* Enteritidis when both technologies were used, resulting in more than 4 log reduction in *Salmonella* Enteritidis in only two minutes of treatment time. While several of these technologies have demonstrated efficacy in sanitizing the exterior of shell eggs, none are currently in use on a large scale.

**Egg products**

Under the Egg Products Inspection Act of 1970, all egg products (liquid, dried or frozen) must be pasteurized. The required minimum temperatures and holding time for different egg products are outlined in the Code of Federal Regulations and summarized in Table 1.7. The Act specifies the target of egg pasteurization as a “salmonella negative product” (United States Food and Drug Administration 1970). Though it covers a wide range of egg products, the Egg Products Inspection Act offers no guideline for whole shell eggs.
Table 1.7 Pasteurization guidelines for selected egg products

<table>
<thead>
<tr>
<th>Product</th>
<th>Target temperature (°C)</th>
<th>Required holding time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole egg</td>
<td>60</td>
<td>3.5 min</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>60</td>
<td>6.2 min</td>
</tr>
<tr>
<td></td>
<td>61.1</td>
<td>3.5 min</td>
</tr>
<tr>
<td>Albumen</td>
<td>55.6</td>
<td>6.2 min</td>
</tr>
<tr>
<td></td>
<td>56.7</td>
<td>3.5 min</td>
</tr>
<tr>
<td>Dried albumen (not previously pasteurized, moisture 6%)</td>
<td>54.4</td>
<td>7 – 10 days</td>
</tr>
</tbody>
</table>

*aModified from Froning et al. 2001.

**Shell eggs**

In 2005, the National Advisory Committee on Microbiological Criteria for Foods published a document stating that the FDA requirement for “pasteurization” of shell eggs calls for a minimum 5 log reduction of *Salmonella* in intact eggs (National advisory committee on microbiological criteria for foods 2005). This requirement applies regardless of the technology used to treat eggs. Several studies have been published regarding treatments for enhancing the safety of shell eggs. The main areas of study have been heat, irradiation, microwave, and ozone. These methods will be discussed individually in more detail.

**Heat Pasteurization**

The practice of heat-treating eggs dates back at least to 1943, when a researcher from Missouri coined the term “thermostabilization.” The process involved heating of eggs immersed in oil at temperatures of 54.4 – 57.7°C for 16 minutes (Goresline et al. 1950). The main objective of thermostabilization was to reduce loss due to spoilage, but
it was also reported to produce eggs that maintained superior quality for up to eight months of storage (Funk et al. 1954). The practice did not gain widespread approval due to loss of functionality, primarily manifested as increased whipping times and reduced angel cake volume of treated eggs, especially those heated at 56.6°C or above (Carlin and Foth 1952).

Heat pasteurization is currently the only method in use to produce pasteurized shell eggs for retail sale. These eggs are produced by immersion in heated water to achieve a targeted time/temperature combination. The first pasteurized whole eggs to enter the market (in 1996) were produced by Michael’s Foods under the brand name Crystal Farms (Mermelstein 2001). Michael’s also sells unpasteurized shell eggs and other egg products under this brand. In 1998, Davidson’s Safest Choice pasteurized eggs were introduced by Pasteurized Eggs Corp., now National Pasteurized Egg, Inc. (Mermelstein 2001). The process used for the production of Davidson’s eggs is based on a number of patents obtained by John Davidson (Davidson 2003, Davidson 2000), and because it is patented, few details are known about the time/temperature combinations that are used commercially. There have been numerous other studies conducted investigating the effects of heat pasteurization on shell eggs, which will be discussed.

In one of the earlier studies, conducted in 1995, eggs were inoculated in the yolk with low levels (10^1-10^3 CFU) of *Salmonella* Enteritidis. The eggs were heated in 57°C water for 25-30 minutes, with authors claiming that more intensive treatments would result in negative effects on egg quality. They found, however, that the parameters used were not severe enough to eliminate the inocula (van Lith et al. 1995). Hou and colleagues inoculated eggs in the yolk with high levels (10^7 CFU) of *Salmonella*
Enteritidis and heated at 57°C for 25 minutes. This treatment resulted in a 3-log decrease of *Salmonella* Enteritidis, but authors noted that moving eggs to a hot air oven for holding after come-up time (CUT, approximately 15 minutes) allowed for total reductions of up to 7-log CFU without apparent adverse effects on egg quality (Hou et al. 1996). Similar results were obtained in comparable experiments conducted by another group (Stadelman et al. 1996). Further investigation was conducted by Schuman, who reported complete inactivation of *Salmonella* Enteritidis (10^8 CFU initial inoculum) after 65-75 minutes of immersion heating at 57°C (Schuman et al. 1997). However, notable quality defects were reported in eggs heated at higher temperatures or for long periods of time (greater than 30 minutes), most significantly, a marked increase in the opacity of albumen (Schuman 1996).

In addition to heating by immersion, the use of hot air has also been explored. One of the main drawbacks of using this method is the long CUT it requires, (Hou et al. 1996, Stadelman et al. 1996), leading to increased overall treatment time. Brackett and colleagues reported complete inactivation of 10^8 CFU *Salmonella* Enteritidis per egg in 70 minutes of treatment at 57.2°C, but did not address quality of treated eggs (Brackett et al. 2001). After treating eggs at 55°C for up to 180 minutes, Hank and associates reported no significant differences in physical or nutritional quality over a 56-day storage period when compared to untreated eggs (Hank et al. 2001).

**Irradiation**

Investigation into the use of irradiation against *Salmonella* in egg products started in the 1950s. While the microbiological results of these experiments were promising,
authors observed the production of unpleasant off-odors as well as loss of yellow color in the yolk (Ball and Gardner 1968). More recent studies have been conducted using gamma, beta (e-beam) or x-ray irradiation. The greater portion of the research conducted has utilized gamma irradiation. It has been reported that doses as low as 2 kGy are effective at completely eliminating *Salmonella* Enteritidis from eggshells and shell membranes (Tellez et al. 1995). The drawback of this technology remains the detrimental effects that are produced in treated eggs. Several authors have observed a significant decrease in Haugh units resulting from doses as low as 0.5 kGy (Tellez et al. 1995, Ma et al. 1993, Ma et al. 1990, Al-Bachir and Zeinou 2006). Yolk discoloration and weakening of both eggshells and vitelline membrane have also been reported (Ma et al. 1986, Dvorák et al. 2005). In sensory analysis of irradiated eggs served soft-boiled, panelists were able to differentiate treated and untreated samples consistently, (Ma et al. 1990), but in a sensory study of mayonnaise prepared with irradiated eggs, no differences could be detected (Al-Bachir and Zeinou 2006). Conversely, some authors have observed an increase in functionality of irradiated albumen, leading to increased whip and angel cake volumes (Ma et al. 1993, Ma et al. 1990, Ma et al. 1986). Irradiation with electron beam at levels of up to 4 kGy was shown to produce a maximum reduction of *Salmonella* Enteritidis (inoculated in albumen) of 3.5-log (Wong and Kitts 2003). This treatment resulted in a loss of Haugh units, reduced yellow coloration of yolks, and reduction of vitelline membrane integrity regardless of dose. Treatment with a maximum of 1.5 kGy using x-rays led to at least 4-log reduction of *Salmonella* Enteritidis inoculated in yolk with no apparent color changes (Serrano et al. 1997), but albumen quality was not addressed. While irradiation at levels of up to 3 kGy was approved by the FDA for use
on shell eggs in 2000, it is not currently being used on an industrial scale due to factors including cost, adverse quality effects and poor acceptance of irradiation on the part of consumers (United States Food and Drug Administration 2000b).

Microwave

More recently, there has been investigation into the use of microwave heating as a method for pasteurization of shell eggs. Studies in this area generally focus on attaining the target time/temperature combinations prescribed by the USDA Food Safety and Inspection Service for albumen and yolk separately. Researchers report that it is possible to pasteurize the yolk of intact eggs without raising the temperature of albumen above that required for its pasteurization, but this work has been conducted only on uninoculated eggs (Sivaramakrishnan 2007, Dev et al. 2008). Microwave technology has not been approved for commercial pasteurization of shell eggs.

OZONE

General characteristics

Ozone is a triatomic oxygen molecule arranged to form an obtuse angle (Horvath et al. 1985). It is liquid above approximately 80 K (Brown et al. 1955, Jenkins and DiPaolo 1956) and boils at 161 K (Horvath et al. 1985). The reduction potential of ozone is 2.07 V, qualifying it as one of the strongest known oxidizers. In the gaseous state, ozone is denser than air (Horvath et al. 1985) and colorless at low concentrations. It possesses a distinct odor described alternately as “fresh” or “fishy” which is detectable by humans at concentrations as low as 0.02 ppm (Horvath et al. 1985). Under natural
circumstances, small amounts of ozone are generated in the earth’s atmosphere by the action of short-wave UV light (<300 nm) on molecular oxygen. It has been noted that the formation of stratospheric ozone confers a benefit to the biosphere by absorbing a not insignificant amount of UV light in the range that is most damaging to proteins and nucleic acids (Horvath et al. 1985). Ozone is extremely reactive, with a half life in the gaseous phase of approximately 12 hours (Horvath et al. 1985), in water, half life is reduced to only 20-30 minutes (depending on water source, purity, temperature, etc.) (Kim et al. 2003). Because it is capable of reacting with a number of substances, including metals and organic compounds, the stability of ozone is greatly dependent on the materials used to contain it, the presence of organic contaminants, and other factors including temperature and pH, with decreasing stability at increased temperatures and pH (Kim 1998).

Ozone pollution

While the presence of ozone is expected in earth’s stratosphere, when found in the troposphere, it is considered a pollutant. Ozone is produced in the troposphere by the interaction of sunlight and volatile organic compounds or nitrogen oxides. Levels of tropospheric ozone vary depending on time of day, season and location, with daily and annual peaks generally observed at the sunniest part of the day (Heagle 1989) and in the spring months (Vingarzan 2004). Common levels of ozone have been reported to fall in the range of 20 – 250 ppb depending upon the preceding factors (Heagle 1989, Sanderman 1996); this range represents an increase in atmospheric ozone of at least
100% during the last century (Vingarzan 2004). The increase has been commonly attributed to the rising use of automobiles in this time period.

Levels of ozone in the troposphere have raised concerns for a number of reasons, principally the possible manifestations of exposure to plant and human health. Ozone levels consistent with those that would occur naturally have been demonstrated to reduce crop yield by more than ten percent (Heagle 1989), and to make plants more susceptible to subsequent stressors (Sanderman 1996). Ozone pollution has also been linked to damage of coniferous trees in the northern hemisphere. While deleterious effects on human health have long focused on the induction of respiratory distress, elevated ozone levels can also precede vasoconstriction, causing a rise in blood pressure (Brook et al. 2002), and have been linked to increased risk of myocardial infarction in humans (Ruidavets et al. 2005).

**Ozone production**

As a consequence of its reactivity, ozone cannot be stored for significant periods, and so it must be generated as needed. Ozone gas can be purposely generated using a number of methods. These include photochemical procedures, which employ UV light but generally result in low ozone concentrations; electrolysis of water to produce ozone and hydrogen gas; and corona discharge. Corona discharge is the most common method in use and is capable of producing relatively high concentrations of ozone. In this method, gas (air or dry oxygen) is passed between two electrodes coated with a dielectric material separated by a discharge gap. A high energy discharge across the gap splits molecular oxygen into its atomic form. Atomic oxygen spontaneously combines with
molecular oxygen to form triatomic ozone (Horvath et al. 1985). When oxygen is used as a feed gas, as opposed to air, higher levels of ozone are subsequently produced. Once produced, ozone can be used in the gaseous state or sparged into water to produce aqueous ozone for rinsing and washing applications.

**Ozone decomposition**

The decomposition of ozone yields a number of oxidative radicals, including the superoxide anion radical and hydroperoxide radical, which subsequently gives rise to the hydroxyl radical (Kim 2003). The hydroxyl radical is incredibly reactive and its interaction with cellular components has been credited for much of the antimicrobial action of ozone. Radical reactions continuously self propagate until a quencher, or inhibitor is encountered, at which point reaction ceases. Because radicals are known to react very quickly, penetration of ozone can be a problem in some applications.

**Ozone measurement**

The high redox potential of ozone often allows for its use in small amounts and contributes to its rapid decomposition under many circumstances. The combination of these factors makes accurate measurement of ozone levels particularly difficult. As with its generation, there are several methods available for the determination of ozone concentration. Historically, the most reliable and widely used method for the determination of ozone in the aqueous phase has been the indigo method. This procedure is based on spectrophotometric determination of the decolorization of indigo trisulfonate upon its reaction with ozone (Bader and Hoigne 1981). Ozone acts by disrupting the sole
carbon-carbon double bond present in the indigo molecule (Figure 1.3). The indigo method is accurate within 2% (Grunwell et al. 1983) and is still often used to measure ozone in the aqueous phase. For quantification of gaseous ozone, the most common and trusted method is based on UV light. It works by measuring the absorbance of a gas sample exposed to UV at 254 nm (Dunlea et al. 2006). Many commercially available ozone monitors use this technology, which is appropriate for a wide range of ozone concentrations and allows continuous, near-real time quantification of ozone residuals.

**Figure 1.3 Oxidation of indigo trisulfonate by ozone**
Modified from (Grunwell et al. 1983)

**Special considerations**

Due to the strong oxidizing power of ozone, there are several considerations that must be taken into account to ensure its safe utilization. Human exposure to ozone above very low levels can lead to a number of negative effects. At low levels, ozone is a respiratory irritant which can cause headache, coughing, dizziness and nausea. Exposure for long periods of time or to higher levels of ozone (e.g., 6 ppm), can lead to pulmonary edema. In this case, inflammation causes obstruction to the entry of alveoli and/or reduction in alveolar volume, leading to diminished breathing capacity (Horvath et al.
Repeated exposures to ozone can result in permanent lung damage (Scheel et al. 1959). Though the respiratory system is the primary site of action in humans, other effects, including vision loss have been reported as well (Lagerwerff 1963). Standards set forth by the Occupational Safety and Health Administration of the United States specify that workers may not be exposed to concentrations exceeding 0.1 ppm for extended periods of time, or 0.2 ppm for short term exposure (United States Department of Labor, Occupational Safety and Health Administration 2004). In order to avoid inadvertent exposure, material selection and equipment maintenance are particularly important. Ozone reacts with several commonly used materials, including rubber and plastic.

**Antimicrobial activity**

Ozone possesses a wide antimicrobial spectrum. Its efficacy has been demonstrated against both Gram-positive and Gram-negative bacteria (Güzel-Seydim et al. 2004, Ingram and Haines 1949); bacterial spores (Ishizaki et al. 1986, Khadre and Yousef 2001); fungi (Oztekin et al. 2006, Allen et al. 2003, Palou et al. 2001); viruses (Kim et al. 1980, Roy et al. 1981) and protozoa (Khalifa et al. 2001). There are several potential sites of action within the cell that could be responsible for the lethality of ozone. Early studies hypothesized that reaction with enzymatic systems interfered with cellular respiration (Ingram and Haines 1949). More recently, the focus has been on the interaction of ozone with the unsaturated lipids of the cell membrane (Thanomsub et al. 2002, Victorin 1992). Membrane damage induced by exposure to ozone has been demonstrated to result in leakage of cellular components followed by cell death (Scott
and Lesher 1963). Membrane damage also allows ozone to penetrate into the cell, where it has been reported to cause DNA strand breaks (Ishizaki et al. 1987). Damage to nucleic acids has been reported to be the major cause of viral inactivation by ozone (Kim et al. 1980, Roy et al. 1981). In bacterial spores, significant damage to the spore coat has been observed (Khadre and Yousef 2001). One point of contention among researchers is whether the effects observed are attributable to the reactions of molecular ozone itself or to the decomposition products that are produced by its reversion to molecular oxygen. Among the decomposition products produced by ozone is the hydroxyl radical, the strongest known oxidizer, which leads many authors to believe that decomposition products are responsible for antimicrobial effects (Block 2001). However, in a study conducted by Hunt and Marinas, radical scavenging compounds were added to treatment media and this addition did not have a significant effect on the reduction of *E. coli* population by ozone treatment (Hunt and Marinas 1997). This finding suggests that molecular ozone may play a significant role in bacterial inactivation. However, in a study conducted on *Bacillus subtilis* spores, hydroxyl radicals were found to be principally responsible for inactivation (Cho et al. 2002).

**Antimicrobial considerations**

Several factors can affect the efficacy of ozone against microorganisms. The suspending medium used for treatment plays a very important role in the efficacy of ozone. Most significantly, the presence of organic material, especially proteins and fats, increases demand for ozone because of its tendency to react with these substances (Güzel-Seydim et al. 2004, Ingram and Haines 1949). As previously mentioned, ozone
stability decreases with increasing pH. If molecular ozone reactions are necessary for inactivation, a low pH is desired; however, higher pH has been demonstrated to encourage formation of hydroxyl radicals, contributing to spore inactivation (Cho et al. 2002). Increased moisture also seems to enhance killing by gaseous ozone. This effect was observed in the treatment of barley grains to inactivate fungi (Allen et al. 2003). In a similar study conducted on wheat, both increased water activity and increased treatment temperature (from 10 to 40°C) led to greater inactivation of fungal spores (Wu et al. 2006). Increased relative humidity has been demonstrated to aid in bacterial spore inactivation (Ishizaki et al. 1986).

Use in food

Ozone has been used for treatment of drinking water since the late 1800s. It is still widely used for this purpose not only as a disinfectant, but also to precipitate heavy metal contaminants (Horvath et al. 1985). In 1982, the FDA gave ozone generally recognized as safe (GRAS) status for use in the treatment of bottled water (Anonymous 1982). In 2001, this status was extended for use in food products, where ozone is considered a food additive (Anonymous 2001). As a result, use under good manufacturing practices is now unlimited. When compared to other sanitizers, notably chlorine, ozone is an attractive choice because its decomposition (to molecular oxygen) does not leave behind any potentially harmful byproducts.

Several researchers have investigated the use of ozone gas as a fumigant for grain products, with favorable outcomes on wheat (Wu et al. 2006), corn (Kells et al. 2001), and barley (Allen et al. 2003, Kottapalli et al. 2005). In addition to the inactivation of
fungal cells and spores, ozone has also been used for detoxification of several mycotoxins. Short ozone treatments (2% by weight for 15 seconds) have been demonstrated to reduce the toxicity of several mycotoxins, including aflatoxin, ochratoxin and patulin suspended in liquid medium (McKenzie et al. 1997). While aflatoxins B$_1$ and G$_1$ were degraded by 2% ozone treatment (5 minutes), aflatoxins B$_2$ and G$_2$ required treatment with 20% ozone. The relative susceptibility of toxins B$_1$ and G$_1$ has previously been reported in a study using peanut and cottonseed meal (Dwarakanath et al. 1968). Patulin suspended in diluted apple juice was rendered undetectable by HPLC after less than one minute of ozone treatment (Cataldo 2008).

Fresh produce is one of the most promising areas for ozone application as the sanitizer can be used for control of pathogens as well as for shelf life extension through restriction of fungal growth. Storage with 0.3 ppm ozone increased the shelf life of citrus fruits by one week and was effective against organisms that exhibit resistance to conventional fungicides (Palou et al. 2001). Shelf life of blackberries was extended by as much as 12 days without significant effect on anthocyanin content (Barth et al. 1995). A Turkish study reported the efficacy of short ozone treatments to kill Salmonella Enteritidis on cherry tomatoes (Das et al. 2006), while a Spanish study reported reduction of natural flora and favorable sensory outcomes for whole and sliced Thomas tomatoes (Aguayo et al. 2006).

Numerous studies have been conducted to investigate the use of ozone during storage of fresh fish. Authors have reported significant increases in shelf life of seafood products including oysters, megrim, turbot and sardines when these products are stored in the presence of gaseous ozone or on ice made from ozonated water (Campos et al. 2006,
Campos et al. 2005, Losada et al. 2004, Pastoriza et al. 2008, Rong et al. 2010). Treatment with less than 0.3 ppm ozone was reported to improve sensory outcomes in a study on fresh scad (Da Silva et al. 1998).

Though research regarding the applicability of ozone has been conducted in a number of food systems, its use is not universal. While ozone is an extremely effective antimicrobial in aqueous solution, the presence of food components can greatly reduce its bacteriocidal activity. Both proteins and fats convey a protective effect on microbes, shielding them from the action of ozone (Güzel-Seydim et al. 2004). Additionally, the oxidative action of ozone can result in detrimental effects to treated food products when it reacts with the product surface. This may result in increased lipid oxidation rates and bleaching of susceptible pigments, depending on the particular product. Detrimental effects have been observed in a number of products, including alfalfa sprouts and pepper flakes (Akbas and Ozdemir 2008b, Wade et al. 2003) as well as ground beef and steak (Pohlman et al. 2002, Greer and Jones 1989). A summary of ozone use for decontamination of different foods is presented in Table 1.8.

Table 1.8 Ozone treatment for decontamination of food products

<table>
<thead>
<tr>
<th>Product</th>
<th>Treatment (Phasea, level, time)</th>
<th>Microbial target</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken skin</td>
<td>G, &gt;2000 ppm, 30 min</td>
<td>Salmonella Infantis, Pseudomonas aeruginosa</td>
<td>&lt; 1-log reduction (both organisms), no negative sensory outcomes</td>
<td>(Al-Haddad et al. 2005)</td>
</tr>
<tr>
<td>Beef</td>
<td>A, 95 mg l⁻¹, spray</td>
<td>E. coli O157:H7, S. Typhimurium</td>
<td>Not more effective than spraying with plain water</td>
<td>(Castillo et al. 2003)</td>
</tr>
<tr>
<td>Scad (fish)</td>
<td>Gaseous, &lt; 0.03 g l⁻¹, 60 min</td>
<td>Mixed inoculum</td>
<td>&gt; 2-log reduction, adverse effects on lipid oxidation or sensory</td>
<td>(Da Silva et al. 1998)</td>
</tr>
</tbody>
</table>
Table 1.8 continued

<table>
<thead>
<tr>
<th>Food</th>
<th>Treatment</th>
<th>Concentration</th>
<th>Time</th>
<th>Microorganisms/Flora</th>
<th>Reduction</th>
<th>Source(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mussels (shucked)</td>
<td>A</td>
<td>1 mg l⁻¹, 60-90 min</td>
<td>Natural flora</td>
<td>0.7 – 2.1-log reduction</td>
<td>(Manousaridis et al. 2005)</td>
<td></td>
</tr>
<tr>
<td>Pistachios (in-shell)</td>
<td>G</td>
<td>1 ppm, 240 min</td>
<td>E. coli, Bacillus cereus</td>
<td>&gt; 2.5-log reduction (both organisms), no increase in rancidity</td>
<td>(Akbas 2006)</td>
<td></td>
</tr>
<tr>
<td>Figs (dried)</td>
<td>G</td>
<td>5 or 10 ppm, 3 or 5 hours</td>
<td>Natural flora</td>
<td>1.46-log reduction coliform count</td>
<td>(Oztekin et al. 2006)</td>
<td></td>
</tr>
<tr>
<td>Figs (dried)</td>
<td>G</td>
<td>1 ppm, 360 min, G, 5-9 ppm, 360 min</td>
<td>E. coli, B. cereus (cells), B. cereus (spores)</td>
<td>3.5-log reduction (both), 2-log reduction</td>
<td>(Akbas and Ozdemir 2008a)(Akbas and Ozdemir 2008b)</td>
<td></td>
</tr>
<tr>
<td>Apples (surface)</td>
<td>A</td>
<td>22-25 mg l⁻¹, 3 min</td>
<td>E. coli O157:H7</td>
<td>2.6-log reduction</td>
<td>(Achen and Yousef 2001)</td>
<td></td>
</tr>
<tr>
<td>Cherry tomato</td>
<td>G</td>
<td>10 mg l⁻¹, 10-20 min</td>
<td>S. Enteritidis</td>
<td>~7-log reduction</td>
<td>(Das et al. 2006)</td>
<td></td>
</tr>
<tr>
<td>Tomato (sliced)</td>
<td>G</td>
<td>4µl l⁻¹, 30 min every 3 hours up to 15 days</td>
<td>Natural flora</td>
<td>1.1-1.2-log reduction bacteria, 0.5-log reduction fungi, no off flavors produced</td>
<td>(Aguayo et al. 2006)</td>
<td></td>
</tr>
<tr>
<td>Lettuce</td>
<td>A</td>
<td>&gt; 10 mg ml⁻¹, 10 min</td>
<td>Natural spoilage flora</td>
<td>1.1-log reduction</td>
<td>(Garcia et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>Green pepper</td>
<td>A</td>
<td>8 mg l⁻¹, 25 min</td>
<td>E. coli O157:H7</td>
<td>7-log reduction (5g)</td>
<td>(Han et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>Green pepper (cut)</td>
<td>A</td>
<td>0.3-3.95 mg l⁻¹, 20 sec – 30 min</td>
<td>Natural flora</td>
<td>Not more effective than plain water</td>
<td>(Ketteringham et al. 2006)</td>
<td></td>
</tr>
<tr>
<td>Potato</td>
<td>A</td>
<td>5 ppm, 1 min</td>
<td>Yersinia enterocolitica, Listeria monocytogenes</td>
<td>1.6-log reduction, 0.8-log reduction</td>
<td>(Selma et al. 2006)</td>
<td></td>
</tr>
<tr>
<td>Baby carrots</td>
<td>G</td>
<td>7.6 mg l⁻¹, 15 min</td>
<td>E. coli O157:H7</td>
<td>2.6-log reduction</td>
<td>(Singh et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>Cantaloupe (cut)</td>
<td>G</td>
<td>10,000 ppm, 30 min</td>
<td>Salmonella spp.</td>
<td>2.8-log reduction (12.6 cm²)</td>
<td>(Selma et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>Alfalfa sprouts</td>
<td>A</td>
<td>5-23 ppm, 2 min</td>
<td>L. monocytogenes</td>
<td>&lt; 1-log reduction, significantly reduced sensory quality</td>
<td>(Wade et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>Black pepper</td>
<td>G</td>
<td>1 ppm, 120 min</td>
<td>E. coli</td>
<td>~7-log reduction</td>
<td>(Emer et al. 2008)</td>
<td></td>
</tr>
</tbody>
</table>

*G = gaseous ozone, A= aqueous ozone*
**Ozone and eggs**

The use of ozone to enhance the safety of eggs has been addressed in a limited number of previous studies. In 2003, Davies and Breslin reported on the use of gaseous ozone to sanitize eggshells inoculated with *Salmonella* Enteritidis. They found that ozone was ineffective at low relative humidity and “partially” effective at high humidity, but concentrations used were not given (Davies and Breslin 2003a). In 2005, Rodriguez-Romo and Yousef reported a decrease of 5.9-log *Salmonella* Enteritidis on egg shells with ozone treatment and further synergistic inactivation by sequential application of UV and ozone (Rodriguez-Romo and Yousef 2005). Work on internal decontamination of eggs by the same group provided promising results (Rodriguez-Romo 2004).

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Chapter 2

Inactivation of *Salmonella enterica* Serovar Enteritidis in Shell Eggs by Sequential Application of Heat and Ozone and Physical Quality of Eggs Treated with this Process

ABSTRACT

The aim of this study is to assess the contribution of ozone to lethality of *Salmonella enterica* serovar Enteritidis in experimentally inoculated whole shell eggs when sequentially treated with heat and gaseous ozone in pilot-scale equipment, and to verify the quality of eggs treated with this process. Whole shell eggs were inoculated with small populations of *Salmonella* Enteritidis (8.5x10^4 – 2.4x10^5 CFU per egg) near the egg vitelline membrane. Eggs were subjected to immersion heating (held in water bath at 57°C for 21 minutes), ozone treatment (vacuum at 67.5 kPa, followed immediately by ozonation at a maximum concentration of ~140 g ozone m⁻³ and pressure of 184 - 198 kPa for 40 minutes) or a combination of both treatments. Survivors were detected by an enrichment process or enumerated using modified most-probable number technique. Ozone, heat and combination treatments inactivated 0.11, 3.1, and 4.2 log *Salmonella* Enteritidis per egg, respectively. Sequential application of heat and gaseous ozone was significantly more effective than either heat or ozone alone. The demonstrated synergy between these treatment steps should produce safer shell eggs than a comparable heat treatment alone. Quality of untreated and ozone-treated eggs (un-inoculated) was
investigated over a period of six weeks of storage at either 4 or 25°C. Eggs were tested on a weekly basis for common markers of physical quality including weight, Haugh units, yolk index, albumen pH, yolk and albumen color. At 4°C, differences between treated and untreated eggs were negligible, but at 25°C, treated eggs retained their physical quality better than untreated eggs. These findings are significant because shell eggs are the most common vehicle for human infection by *Salmonella* Enteritidis. Many cases of egg-related salmonellosis are reported annually despite efforts to reduce contamination, including thermal pasteurization of shell eggs and egg products. Treatment with ozone-based combination should produce shell eggs safer than those treated with heat alone, without compromising egg quality.

**INTRODUCTION**

*Salmonella* is one of the most prevalent pathogens associated with foodborne illness. It is the cause of an estimated 1.4 million illnesses annually in the United States alone (Lynch et al. 2006). In recent decades, the proportion of salmonellosis attributed to *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*Salmonella* Enteritidis), relative to other *Salmonella* serovars, has increased. According to a 2001 report (Guard-Petter 2001), Enteritidis is the most prevalent serovar implicated in verified cases of salmonellosis worldwide. The most common food source of *Salmonella* Enteritidis is eggs. Shell eggs are commonly contaminated in a vertical manner during egg formation in the infected hen oviduct, or horizontally contaminated via migration of salmonellae through the egg shell after contact with substances such as hen feces (Lynch et al. 2006, Guard-Petter 2001, United States Department of Health and Human Services, Centers for
Disease Control and Prevention 2003, Grijspeerdt et al. 2005). Internally contaminated eggs, obtained from naturally infected hens, may contain $\leq 10$ *Salmonella* Enteritidis per egg and the pathogen is most likely localized outside the vitelline membrane or in the albumen surrounding it (Humphrey et al. 1991, Poppe et al. 1992, Humphrey 1994). Consumption of eggs that are raw or undercooked and pooling of eggs (a common practice in restaurants and institutional settings) can increase risk of illness (Lynch et al. 2006, United States Department of Health and Human Services, Centers for Disease Control and Prevention 2003).

The widespread risk of salmonellosis has led to the introduction of numerous strategies to decrease egg contamination and resulting illnesses. These strategies include consumer education regarding safe egg handling and cooking practices and implementation of quality assurance programs in egg production facilities (Mumma 2004). While these efforts have succeeded in reducing the number of cases of salmonellosis from the peak of 3.9 per 100,000 people reached in 1994, data for 2003 showed 1.7 cases per 100,000 people, indicating that a serious risk remains (Braden 2006).

Ozone is a colorless gas consisting of three oxygen atoms. It is a strong oxidizing sanitizer and has been approved by the US Food and Drug Administration (FDA) for use in foods (United States Food and Drug Administration 2006). Application of ozone either in gaseous form or via oxidized water has shown promise as an antimicrobial agent against a number of pathogens in several food systems including lettuce and beef (Novak and Yuan 2004, Selma et al. 2007). The efficacy of gaseous ozone against *Salmonella* Enteritidis on the surface of whole eggs has also been demonstrated, as has ozone’s
ability to penetrate intact egg shells (Rodriguez-Romo 2004, Rodriguez-Romo et al.
2007). The present research investigates the possibility of reducing or potentially
eradicating Salmonella Enteritidis in whole shell eggs via treatment with a novel process
involving immersion heating and application of high levels of ozone under pressure.
Previous research has been aimed at assessing the effectiveness of combination processes
and defining process parameters (Rodriguez-Romo 2004).

Previous research investigating the sanitation of shell eggs has involved
technologies including immersion heating and gamma irradiation. While these
treatments have achieved varying levels of success at reducing internal Salmonella
Enteritidis contamination, they have done so at the expense of egg quality. In the current
study, emphasis has been placed on possible industrial application of this technology by
simulating conditions of natural contamination through use of lower inoculum level and
scaled-up treatment setup, as well as detailed investigation of treatment effects on egg
quality. See Appendix A for additional information regarding initial investigation of
process parameters using pilot-scale equipment setup.

MATERIALS AND METHODS

Egg Preparation

Unfertilized, unwashed eggs were obtained from the farms of Hemmelgarn &
Sons, Inc. (Coldwater, OH). Eggs were stored at 4°C and were used within two weeks of
laying. All eggs were measured with Vernier calipers (Manostat Co., Merenschwand,
Switzerland) and only those with a width of 45-46 mm were used for experimental
treatments. Selected eggs were stored at room temperature for approximately two hours
before being scrubbed by hand individually with a plastic brush under cool running tap water. Washed eggs were soaked in ethanol (70% vol/vol) for 30 minutes to sanitize shells. Sanitized eggs were then placed in previously sterilized egg trays and allowed to dry at room temperature for 40 minutes, approximately, prior to inoculation.

**Culture Preparation**

*Salmonella* Enteritidis ODA 99-30581-13, isolated from an egg source, was provided by the Ohio Department of Agriculture (Reynoldsburg, OH). The pathogen was cultured in tryptic soy broth (TSB; Criterion, Hardy Diagnostics, Santa Clara, CA) incubated at 37°C for 24 hours. Thereafter, 0.15 ml of overnight culture was transferred to 150 ml MacConkey broth (Alpha Biosciences, Baltimore, MD), and incubated for additional 24 hours in an orbital shaker (Lab-Line, Mumbai, India) set at 37°C and 175 rpm. Duplicate 45-ml aliquots of this culture were centrifuged at 4°C and 3020 x g for 10 minutes. Cell pellets were resuspended in 2.5 ml chilled phosphate buffer (0.2 g mol⁻¹, pH 7) and combined for a final concentration of approximately 10¹¹ CFU ml⁻¹; this cell suspension was diluted as needed to achieve proper cell concentration for desired inoculum level.

**Inoculation**

Shell eggs were inoculated with *Salmonella* as described earlier (Rodriguez-Romo 2004). Briefly, sanitized eggs were punctured in the approximate center of the blunt side using a sterile 1.5 inch/20-gauge needle attached to a disposable 1 ml syringe (Becton Dickinson, Franklin Lakes, NJ). *Salmonella* Enteritidis cell suspension (10 μl,
~10^7 CFU ml⁻¹, verified by direct plating) was introduced into eggs near the vitelline membrane using a repeating pipette (Yellow Springs Instrument Co., Yellow Springs, OH) with rubber stopper affixed 5 mm from needle point. Inoculation site was wiped with ethanol (70% vol/vol) and allowed to dry for one minute. Holes were then sealed using Teflon tape (Cole-Parmer, Vernon, IL). Resulting internal inoculation level of Salmonella Enteritidis was 8.5x10⁴ – 2.4x10⁵ CFU per egg.

**Heat Treatment**

Inoculated eggs were placed in a wire basket and heat treated by submersion in a circulating water bath (Thermo Corporation, Waltham, MA) set to 57°C. Total treatment time was 21 minutes. Internal egg temperature was monitored using an un-inoculated egg with thermocouple (Fluke, Everett, WA) wire inserted to depth of placed inoculum. Immediately after treatment, eggs were either stored at 4°C until analysis (for heat alone samples) or transferred to ozonation vessel for further treatment.

**Ozone Treatment**

Eggs were subjected to vacuum (67.5 kPa) prior to ozone treatment. Gaseous ozone was produced from pure oxygen by an ozone generator (Ozonia, Elmwood Park, NJ). The output of this generator is an ozone-oxygen mixture that will be referred to as “ozone gas” or “gaseous ozone”. Generator output was pumped to a custom treatment vessel (Figure 2.1(B)) to reach a maximum ozone concentration of 140 g m⁻³. Ozone concentration was monitored continually using an ultraviolet ozone detector (Mini-Hicon model; IN USA, Inc., Norwood, MA). Introduction of ozone to the treatment vessel
resulted in increased pressure, with the final pressure being 184 – 198 kPa. Once desired ozone concentration and pressure were reached, (come-up time of 10 - 12 minutes), gas inlet was closed and eggs were subjected to static treatment for 40 minutes. At the end of treatment, pressure and ozone were released from the vessel and ozone was destroyed by a thermal destruct unit (Ozonia, Elmwood Park, NJ). Treated eggs were removed from the vessel and held at 4°C for 18 hours to insure exhaustion of ozone residues. A schematic representation of experimental setup is shown in Figure 2.2.

Figure 2.1 Benchtop and pilot-scale vessels for ozonation of shell eggs
A: Benchtop vessel, two egg capacity
B: Pilot-scale vessel, 36 egg capacity
Enumeration of Surviving Cells

Eggs were analyzed 18 hours post-treatment for surviving *Salmonella* Enteritidis. Untreated and ozone-only controls were spread-plated on trypticase soy agar and incubated at 37°C for 48 hours. Eggs treated with heat and ozone-heat combination were analyzed for viable *Salmonella* using a most probable number (MPN) technique that was customized (by increasing sample volume) to detect \( > 0.03 \text{ MPN g}^{-1} \). Tested shell eggs were pooled in groups of two and diluted to \( 10^{-1} \) in TSB (original dilution). Aliquots of the original dilution (100, 10 and 1 ml\(^{-1}\), containing 10, 1, and 0.1 g\(^{-1}\) egg, respectively) were used for MPN analysis. Remaining volume of original dilution was incubated at 37°C for 48 hours before streaking onto xylose lysine desoxycholate agar (XLD; Alpha Biosciences, Baltimore, MD) for confirmation of overall negative results, i.e., when all MPN flasks tested negative. MPN flasks were incubated at 37°C for 24 hours before streaking on XLD agar to confirm presence of *Salmonella*. When typical *Salmonella* colonies on XLD were detected, egg was considered positive for the pathogen.
Physical Quality

Untreated and heat-plus-ozone treated eggs were stored in formed pulp trays at either 4°C or 25°C. Eggs were tested for quality factors immediately after treatment (Day 0) and once per week for a total of six weeks. Two eggs from each storage temperature/treatment combination were tested on each testing date. Eggs stored at 4°C were allowed to warm at room temperature for at least three hours prior to testing to avoid artificially high Haugh unit and yolk index values. The entire experiment was performed in duplicate. Quality testing was conducted as follows.

Egg weight

Eggs were placed in a tared weigh boat and weighed on a laboratory balance (Mettler Toledo, Columbus, OH).

Yolk index

Eggs were broken out onto a flat surface. The width and height of egg yolk was measured using Vernier calipers (Manostat Co., Merenschwand, Switzerland), values were recorded and used to calculate yolk index.

Haugh units

Eggs were cracked into a plastic dish resting on a level surface. Haugh units were measured using a Haugh unit meter (Mattox & Moore, Indianapolis, IN). The formula for Haugh units is:

$$HU = 100 \log(h-.01*5.6745(30w^{.37}-100)+1.9)$$
Where HU = Haugh units, h = thick albumen height and w = weight of the unbroken egg.

Haugh unit meter was placed over the egg with measuring pin positioned approximately half way between the yolk and outer edge of thick albumen, avoiding the chalzae. Measurements were taken in three different areas of the albumen and averaged.

**Albumen pH**

Albumen was separated from the yolk using the egg shell. The separated albumen was placed in a glass beaker and stirred on a magnetic stirrer (Thermix Model 210T, Fisher Scientific, Pittsburg, PA) at low speed for one minute. Albumen pH was measured using a standard pH meter (Accumet model 15, Fisher Scientific, Pittsburgh, PA).

**Yolk and albumen color**

Color of both yolk and albumen was measured on the Hunter LAB scale using a handheld colorimeter (Konika Minolta, Tokyo, Japan). Measurements were taken from three distinct areas of each phase and averaged.

**Statistical Analysis**

Data analysis was performed using SAS v. 9.1.3 software (SAS Institute, Inc., Cary, NC). *Salmonella* inactivation results were compared using an unpaired t-test with probability value <0.05 considered significant. Quality results were compared using analysis of variance.
RESULTS

Salmonella-inoculated shell eggs were exposed sequentially to heat, vacuum, and pressurized ozone gas. Water bath temperature was maintained at 57°C (± 0.5°C). Average internal temperature of eggs upon conclusion of heating step (i.e., after 21 min of egg immersion in the water bath) was 56.2°C (Figure 2.3). A vacuum (67.5 kPa) was attained after ≤ 5 minutes of closing the vessel and starting the vacuum pump. The low-pressure vessel was charged with ozone gas to attain required ozone concentration and pressure; this process was completed in 11-12 min. Pressure within the treatment vessel remained relatively stable throughout treatment (Figure 2.4). A slight decrease in measured ozone concentration was observed; this may be attributed to the inherent instability of ozone molecules or reaction with organic material on egg surface. Sequential treatment of shell eggs with heat, vacuum and ozone was completed in approximately 100 minutes, including come-up time for various treatment steps.
Figure 2.3 Internal egg temperature and water bath temperature during heat treatment of shell eggs.

Figure 2.4 Ozone concentration and pressure inside treatment vessel during treatment of shell eggs.
Heat alone and combination treatments resulted in a significant decrease in *Salmonella* Enteritidis, but this was not the case in eggs subjected to ozone without a prior heating step (Table 2.1). Treatment with ozone alone resulted in only 0.11 log reduction, a difference that is not significant from the untreated control. Heat treatment decreased *Salmonella* population by 3.1 log and heat-ozone combination eliminated 4.2 log. This level of inactivation represents fewer than one viable cell per gram of egg treated with heat-ozone combination. Flasks producing negative MPN results were incubated to confirm presence or absence of *Salmonella* Enteritidis. *Salmonella* was recovered from all eggs treated with ozone alone and heat alone, but only 10 of 18 combination-treated eggs tested positive, indicating *Salmonella* eradication in a considerable portion of those samples (Table 2.1).

**Table 2.1 Inactivation of *Salmonella* Enteritidis in shell eggs after various treatments and total number of treated samples testing positive for surviving salmonellae.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment conditions</th>
<th>Average(a) decrease in log CFU per egg</th>
<th>Positive/total(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated and non-treated</td>
<td>None</td>
<td>0(A)</td>
<td>18/18</td>
</tr>
<tr>
<td>Ozone (O(_3))</td>
<td>([O_3] \sim 140 \text{ g m}^{-3} \text{ (maximum) at 184-198 kPa for 40 min})</td>
<td>0.11(A)</td>
<td>18/18</td>
</tr>
<tr>
<td>Heat</td>
<td>57°C for 21 min</td>
<td>3.1(B)</td>
<td>18/18</td>
</tr>
<tr>
<td>Heat and Ozone</td>
<td>57°C for 21 min; vacuum (67.5 kPa); ([O_3] \sim 140 \text{ g m}^{-3} \text{ at 184-198 kPa for 40 min})</td>
<td>4.2(C)</td>
<td>10/18</td>
</tr>
</tbody>
</table>

\(a\) Averages for 18 treated eggs; initial inoculum was 4.9-5.4 log CFU per egg. Averages with different superscripts are significantly different \(p < 0.05\)

\(b\) Presence of *S*. Enteritidis confirmed by streaking of the positive MPN tube or the extended enrichment on xylose lysine deoxycholate agar.
Quality of treated (heat and ozone combination) and untreated eggs was assessed over the course of six weeks of storage. No differences in the color of yolk or albumen were detected on day zero or any subsequent testing date, regardless of storage temperature ($p > 0.05$). Albumen pH (Figure 2.5) was lower in treated eggs than untreated eggs at every testing date regardless of storage temperature. Albumen pH is known to increase during storage due to the loss of free carbon dioxide, and this increase is generally considered to impart unfavorable quality changes to the egg. While this trend was observed in untreated samples, treated eggs retained a relatively stable value for albumen pH throughout the storage period.

![Figure 2.5 Albumen pH of treated and untreated eggs over six weeks of storage. Difference in pH between treated and untreated samples within storage temperature is significant ($p < 0.05$) at all time points. Error bars represent standard error ($n = 8$).](image)
With the exception of pH values, no significant differences were detected between treated and untreated eggs stored at 4°C. However, in eggs stored at 25°C, differences in yolk index (Figure 2.6) and Haugh units (Figure 2.7) between samples were detected at weeks 4, 5 and 6. Values for yolk index are expected to decline as eggs age, due to water migration from albumen to yolk contents as well as the reduced strength of the vitelline membrane. Haugh units are also known to decrease with increasing age, but the reason for this change is not well understood. The decreasing trend is observable for both indices in untreated samples, but not in treated eggs. Significantly higher values for both of these parameters in treated eggs indicates a superior maintenance of both albumen and yolk quality.

![Figure 2.6 Yolk index of eggs stored at 25°C for up to six weeks.](image)

*Indicates values significantly lower than week 0 ($p < 0.05$). Error bars indicate standard error ($n = 8$)
DISCUSSION

The results of this study clearly express the efficacy of heat and ozone combination treatment to inactivate internal contamination in shell eggs in a synergistic manner, offering greater safety than the use of either process alone. In addition, quality testing demonstrates that this unique combination process does not compromise the quality of shell eggs during the course of normal storage. On the contrary, if eggs are subjected to temperature abuse, yolk and albumen quality are better maintained in eggs that have been subjected to heat plus ozone processing.

Though progress toward safer eggs has been made in recent years, a significant risk still remains. A recent risk assessment conducted by the US Department of Agriculture, Food Safety and Inspection Service, predicted that pasteurization of all shell
eggs resulting in a three log reduction of *Salmonella* Enteritidis populations would decrease the annual number of associated illnesses from 130,000 to 41,000; a reduction of nearly 70% (United States Department of Agriculture, Food Safety and Inspection Service 2005). As demonstrated, a heat-ozone combination process possesses the potential of achieving this goal. Whole egg products pasteurized by heat alone are currently available in the US marketplace (National Pasteurized Eggs 2007). Since their introduction in 2000, there have been no outbreaks associated with pasteurized eggs, but the long heating time necessary to achieve a suitable reduction in salmonellae has been observed to adversely affect the quality and clarity of albumen (Rodriguez-Romo 2004, Schuman 1996). These effects on protein quality lead to increased opacity of egg whites and decreased whip volume, which may be off-putting to consumers or restrict use in whipped products. The use of heat-ozone combination, compared to heating only, allows for a considerable reduction in overall heating time without compromising the safety of treated eggs. This reduction in treatment time may serve to minimize heating’s adverse effects on egg quality, explaining the lack of differences observed in quality tests conducted as part of this study.

We propose that heating shell eggs increased permeability of their membranes to ozone gas. Therefore, application of ozone was effective against internal *Salmonella* only when shell eggs were subjected to heat prior to ozone treatment. Unlike a previous investigation in this laboratory (Rodriguez-Romo 2004), the current study reports the synergy between ozone and heat when a smaller population of *Salmonella* is introduced in eggs. Additionally, eggs were treated with ozone in a larger pilot-scale chamber to simulate industrial application.
Many studies show that when bacteria are found in a complex matrix, thermal inactivation deviates from first-order kinetic (Moore and Madden 2000, Buzrul and Alpas 2007). Diminishing bacterial lethality with extended treatment is often described as death curve “tailing.” Presence of resistant sub-population of the treated bacterium is believed to cause the tailing phenomenon (Moore and Madden 2000). Prior work done on the strain used in this study has demonstrated the presence of a significant tailing effect during egg heating (Rodriguez-Romo 2004). Based on the results of the current study, it is proposed that ozone (which was applied subsequent to thermal treatment) inactivates the most heat-resistant Salmonella sub-population. Results of this study support the feasibility of pasteurizing shell eggs using minimal heating that is followed by a carefully planned ozone treatment.

REFERENCES


Chapter 3

Development of a Heat and Ozone Combination Treatment Yielding Sufficient Inactivation of *Salmonella enterica* Serovar Enteritidis in Egg Yolk to Qualify as a “Pasteurization Process”

ABSTRACT

This study aims to expand on previous work using heat and ozone combination treatments against *Salmonella enterica* serovar Enteritidis in shell eggs. Prior studies have illustrated the promise of this technology to produce safe eggs, but a process suitable to be labeled “pasteurization” has not yet been developed. Whole shell eggs were inoculated with *Salmonella* Enteritidis in the egg yolk, where cells were allowed to grow to high levels (~10⁷ CFU g⁻¹ egg). Eggs were subjected to one of several treatments including i). immersion heating only at varying time/temperature combinations; ii). immersion heating at varying time/temperature combinations followed by vacuum (50.8 kPa) and treatment with ozone (maximum 160 g m⁻³) under pressure (~200 kPa). All treatments tested produced greater than 5 log inactivation, which, according to FDA standards, qualifies all treatments as “pasteurization” processes. There was no significant difference among treatments in the numbers of *Salmonella* Enteritidis inactivated. There were, however, significant differences observed in the visual quality of eggs depending on treatment parameters. Application of ozone subsequent to heating allows for a significant reduction in total heating time, without decreasing process lethality, leading to
eggs with better overall visual quality. More in-depth investigation of egg quality is necessary, but the current study highlights the promise of ozone technology to produce a product with comparable safety and superior quality when compared to commercially available thermally-pasteurized eggs.

INTRODUCTION

Consumption of egg-containing foods is the principal risk factor for infection with Salmonella enterica serovar Enteritidis (Salmonella Enteritidis) in the US (United States Department of Agriculture, Food Safety and Inspection Service 2005). Pasteurization of egg products in liquid, dried and frozen states has been mandatory since the release of the 1970 Egg Products Inspection Act (United States Food and Drug Administration 1970). The implementation of pasteurization has dramatically reduced the estimated number of illnesses associated with these products (United States Department of Agriculture, Food Safety and Inspection Service 2005). However, there is no pasteurization requirement for shell eggs. Illness due to consumption of contaminated shell eggs is still viewed as a serious problem, and is estimated to result in at least 180,000 illnesses annually in the US (United States Department of Agriculture, Food Safety and Inspection Service 2005, Schroeder et al. 2005). In 1999, the President’s Council on Food Safety released an action plan relating to egg safety with a goal of 50% reduction in outbreaks of Salmonella Enteritidis (compared to 1997 levels) by 2005, a goal that was not met. One of the main recommendations of this plan was the implementation of a “kill step” in shell egg processing (President's Council on Food Safety 1999). In a risk assessment published in 2005, the United States Department of Agriculture, Food Safety and Inspection Service
estimated that the implementation of treatments to produce at least three-log reduction in *Salmonella* Enteritidis would result in a reduction of shell egg-associated illness of almost 70% (United States Department of Agriculture, Food Safety and Inspection Service 2005).

Several technologies have been investigated for treatment of shell eggs. Efficacy of gamma irradiation against internal *Salmonella* Enteritidis has been demonstrated (Serrano et al. 1997), and use of this technology for shell egg pasteurization was approved (at levels up to 3 kGy) by the Food and Drug Administration (FDA) in 2000 (United States Food and Drug Administration 2000). However, this technology is not currently being used to produce products for retail sale. Despite its microbiological effectiveness, consumer acceptance is lacking because even low doses of irradiation result in severe quality loss. Detrimental effects include the production of persistent off-odors (Ball and Gardner 1968), discoloration of yolk (Dvorák et al. 2005), weakening of vitelline membrane and shells (Ma et al. 1986), and loss of albumen viscosity (Tellez et al. 1995).

Immersion heating has been recognized by the FDA as a valid method for shell egg pasteurization. The two companies currently employing this method use patented processes, negating the release of standard accepted conditions. Heat pasteurized eggs have been available regionally since 1996 (Mermelstein 2001), and a number of studies have been conducted to explore the efficacy of this method. Generally, thermal pasteurization consists of heating eggs in water at a temperature in the range of 55-58°C for a specified time to ensure appropriate destruction of *Salmonella* Enteritidis. Treatments may be designed around water bath temperature (treatment temperature) and
the total time eggs spend in the water bath (treatment time). Alternatively, internal egg

temperature may be monitored with treatment defined by a “target” internal temperature

and holding time at that temperature, following initial come-up time in the water bath.

Quality deterioration has been observed in eggs heated longer than 30 minutes, total


1995), or to internal temperatures above 57°C (Schuman 1996, Rodriguez-Romo 2004).

Early studies on heat treatment of shell eggs indicated that treatment at 55-57°C

(treatment temperature) for 25-30 minutes total treatment time produced eggs with

quality markers similar to those of untreated samples. However, these treatments resulted

in only approximately 3-log reduction of *Salmonella* Enteritidis (Hou et al. 1996,

Rodriguez-Romo 2004, Perry et al. 2008). Because naturally contaminated eggs are

estimated to contain an average of only 10-100 cells of *Salmonella* Enteritidis (Humphrey

et al. 1991), a 3-log reduction would theoretically provide a significant margin of safety,

but eggs subjected to this treatment could not be labeled as “pasteurized.” According to

standards developed by the National Advisory Committee on Microbiological Criteria for

Foods, pasteurization requires a minimum of 5-log reduction of organism of concern

(National advisory committee on microbiological criteria for foods 2005). In order to

satisfy this requirement, treatment times for thermally pasteurized shell eggs are currently

as long as 180 minutes (Davidson 2003, Davidson 2000). The result of this process is a

safe egg with reduced visual appeal and functional properties.

Previous work in this laboratory has focused on the use of a two-technology

combination process to eliminate *Salmonella* Enteritidis in shell eggs. Briefly, this

process involves a shortened immersion heating treatment followed by application of
vacuum and static treatment with gaseous ozone under pressure. The use of two technologies (heat and ozone) implements a “hurdle” treatment that allows for a less severe heat treatment and offers an added measure of safety against heat-resistant *Salmonella* subpopulations.

Ozone is a compound composed of three oxygen atoms. It is one of the strongest known oxidizers with demonstrated efficacy against a wide range of organisms including bacterial cells (Ingram and Haines 1949, Güzel-Seydim et al. 2004), bacterial spores (Ishizaki et al. 1986, Khadre and Yousef 2001), fungi (Palou et al. 2001, Oztekin et al. 2006, Allen et al. 2003), and viruses (Kim et al. 1980, Roy et al. 1981). When compared to other sanitizers commonly used for food applications, a major advantage of ozone is that it decomposes to molecular oxygen, leaving behind no harmful byproducts.

Ability of ozone gas to penetrate intact egg shells has been demonstrated (Rodriguez-Romo et al. 2007), supporting its applicability for shell egg treatment. Initial work using heat and ozone combinations in a small scale setup demonstrated the strong potential of this approach (Rodriguez-Romo 2004). More recently, pilot-scale equipment was utilized in a process that demonstrated synergy between heat and ozone treatment, resulting in a 4.2 log reduction of *Salmonella* Enteritidis, inoculated near the vitelline membrane (Perry et al. 2008). It is the goal of this study to modify previous treatments in order to produce the requisite 5 log reduction of *Salmonella* Enteritidis. Additionally, eggs have thus far been inoculated in the albumen portion of the egg, mimicking natural internal contamination. Eggs in this study will be inoculated in the yolk in order to simulate a “worst-case scenario.”
MATERIALS AND METHODS

Design of pasteurization treatments

Commercial thermal pasteurization was included in this study as a reference treatment for comparing processes lethality and visual qualities of processed eggs. The commercial process was identified using information from patents owned by National Pasteurized Egg, Inc. (Davidson 2003, Davidson 2000). Three treatments were chosen to correspond with the lower confidence limit of acceptable treatment time/temperature combinations as illustrated in these patents (Figure 3.1). The lower confidence interval was chosen to assure that eggs produced would incur the least possible amount of heat damage while still complying with prescribed treatment limits.

Figure 3.1 Time/temperature combinations required to produce thermally pasteurized eggs as specified in the US patent 6,632,464 (Davidson, 2003)
All treatment parameters from this point on will be given as internal target temperature and holding time at target temperature, disregarding come-up time. The treatments selected were (internal temperature, holding time at target temperature): 54.4°C, 80 min; 51.6°C, 32 min; 57.7°C, 13 min. Un-inoculated eggs were subjected to these treatments and visual quality was assessed (Figure 3.6). The treatment judged to have the best visual quality was selected as the standard for thermal pasteurization comparison in further work. *Salmonella*-inoculated eggs were subjected to the selected treatment and process lethality to the pathogen was assessed as described later.

Heat treatment steps for combination process pasteurization were chosen based on the most severe treatment known by investigators to produce eggs with acceptable visual quality (internal temperature of 56°C for 10 minutes holding). Using this combination, a line was constructed approximately parallel to that describing efficacious treatments in the thermal pasteurization patents (Figure 3.2). From this line, three additional time/temperature combinations were selected. The heat treatment steps chosen for combination pasteurization were (internal temperature, holding time at target temperature): 55°C, 20 min; 56°C, 10 min; 57°C, 5 min; 58°C, 3 min. Each of these heat treatments was paired with a standard ozone treatment to yield the combination pasteurization processes.
Figure 3.2 Selection of heat treatment steps for heat-ozone pasteurization process.

Egg Preparation

Unfertilized, washed Grade AA large eggs were obtained from the farms of Hemmelgarn & Sons, Inc. (Coldwater, OH). Eggs were stored at 4°C until use and were used within two weeks of laying. All eggs were weighed and only those weighing 2 oz ± 5% (56.7 ± 2.8 g) were used for experimental treatments. Selected eggs were stored at room temperature for approximately 18 hours before inoculation to prevent contamination due to internalization of condensation.

Culture Preparation

Salmonella Enteritidis ODA 99-30581-13, isolated from an egg source, was provided by the Ohio Department of Agriculture (Reynoldsburg, OH). Cells were cultured in tryptic soy broth (TSB; Criterion, Hardy Diagnostics, Santa Clara, CA) and incubated at 37°C for 24 hours. Overnight culture was centrifuged at 4°C and 3020 x g
for 10 minutes. Cell pellets were re-suspended in equal volume of chilled phosphate buffer (0.2 mol l⁻¹, pH 7). Washed cell suspension was diluted as needed to achieve proper cell concentration for desired inoculum level.

**Inoculation**

Shell eggs were punctured in the approximate center of the blunt side using a sterile 1.5 inch/20-gauge needle attached to a disposable 1 ml syringe (Becton Dickinson, Franklin Lakes, NJ). *Salmonella* Enteritidis cell suspension (10 μl, 10²-10³ CFU ml⁻¹, verified by direct plating) was introduced into egg yolks using a gas tight syringe coupled to 2 inch/22-gauge removable needle (Hamilton Company, Reno, NV) with rubber stopper affixed 17 mm from needle point. Inoculation site was wiped with ethanol (70% vol/vol) and allowed to dry for one minute. Holes were then sealed using Teflon tape (Cole-Parmer, Vernon, IL). Resulting internal inoculation level of *Salmonella* Enteritidis was ~10¹-10² CFU per egg. Inoculated eggs were incubated at 30°C for 24 hours prior to treatment. The use of this procedure serves two purposes; i) incubation time allows cells to become accustomed to the environment of the yolk, reducing the risk of altered physiology due to change of environment immediately before treatment; and ii) allowing low numbers of cells to grow to desired final population ensures high enough cell density for quantitative results without risk of confounding factors introduced by cell clumping upon inoculation of large numbers of pathogen cells.
**Heat Treatment**

Inoculated eggs were placed onto a custom-made stainless steel rack (Figure 3.3) and heat treated by immersion in a circulating water bath (Thermo Corporation, Waltham, MA) set to approximately one degree higher than target temperature. Temperature in the approximate center of the yolk was monitored using an un-inoculated egg with thermocouple (Fluke, Everett, WA) wire inserted to depth of placed inoculum. Once eggs reached target internal temperature, they were held in the water bath for the prescribed holding time. Immediately after treatment, eggs were either stored at 4°C until analysis (for heat alone samples) or transferred to ozonation vessel for further treatment.

![Figure 3.3 Custom-made stainless steel rack for heat and ozone treatment of shell eggs.](image-url)
Ozone Treatment

Eggs were subjected to vacuum (50.8 kPa) prior to ozone treatment. Gaseous ozone was produced from pure oxygen by an ozone generator (Ozonia, Elmwood Park, NJ). Ozone gas was pumped to a custom treatment vessel to reach a maximum ozone concentration of 160 g m$^{-3}$. Ozone concentration was monitored continually using an ultraviolet ozone detector (Mini-Hicon model; IN USA, Inc., Norwood, MA). Introduction of ozone causes a concurrent increase in pressure within the treatment vessel, with the final pressure being ~187.5 kPa. Once desired ozone concentration and pressure were reached (come-up time of 10 - 12 minutes), gas inlet was closed and eggs were subjected to static treatment for 60 minutes. Pressure levels were maintained throughout this time, while ozone concentration decreased slightly. At the end of treatment, pressure and ozone were released from the vessel and ozone was destroyed by a thermal destruct unit (Ozonia, Elmwood Park, NJ). Residual ozone was flushed from the vessel using compressed air. Treated eggs were removed from the vessel and analyzed immediately for surviving salmonellae.

Enumeration of Surviving Cells

Eggs were analyzed within four hours post-treatment for surviving *Salmonella Enteritidis*. All eggs were pooled in groups of two for homogenization. Inoculated, untreated controls were spread-plated onto trypticase soy agar (TSA) plates which were incubated at 37°C for 48 hours for verification of starting inoculum level. Eggs treated with heat or heat plus ozone combinations were diluted 1:1 in 0.1% peptone water and spread-plated, without further dilution, onto TSA plates. Inoculated plates were incubated
at 37°C for 48 hours and colony formation was observed. For confirmation, colonies growing on TSA were streaked onto xylose lysine desoxycholate agar (XLD; Alpha Biosciences, Baltimore, MD). Small surviving populations of *Salmonella* (i.e., below the detection limit of the plating technique) were detected using an enrichment procedure as follows. Original egg-peptone homogenate was incubated at 37°C for 48 hours. Incubated homogenate was streaked onto XLD plates, and formation of typical *Salmonella* colonies on this medium was indicative of the presence of the pathogen in the sample.

**Heat-ozone combination processing of eggs with variable heat treatment**

Because log reductions observed in the previous experiments were higher than necessary for qualification as pasteurization (Table 3.2), the possibility of a reduction in heating time (for improved quality of final product) was examined. Combinations treatments using a 56°C internal temperature with 0-10 minute holding time were tested. All procedures were identical to those described earlier with the exception of holding time. Three replicates of each combination (using 12 eggs per replicate) were completed.

**Statistical Analysis**

Data analysis was performed using SAS v. 9.1.3 software (SAS Institute, Inc., Cary, NC). Results were compared using a general linear model with treatment as independent variable and log reduction as dependant variable. Probability value < 0.05 was considered significant.
RESULTS

Incubation of inoculated shell eggs at 30°C for 24 hours increased internal levels of *Salmonella* Enteritidis from approximately 10-100 cells per egg to more than $10^7$ cells per gram of egg contents (Figure 3.4). Incubation for 72 hours increased *Salmonella* population to approximately $10^9$ cells per gram. These levels correspond to those observed in another study using a similar inoculation protocol with egg incubation at 23°C (Saeed and Koons 1993).

![Figure 3.4 Growth of Salmonella Enteritidis in egg yolk of inoculated shell eggs incubated at 30°C.](image)

Error bars represent standard deviation (n=6).

**Selection of thermal treatment representing commercial pasteurization**

Representative photographs of eggs subjected to the four heat-only pasteurization treatments are displayed in Figure 3.5. Eggs were compared by researchers on the basis of albumen turbidity. Upon visual inspection, researchers selected 56.1°C, 32 min as the standard thermal treatment for comparison. This treatment produced a 6.96-log decrease.
in *Salmonella* Enteritidis per gram of egg contents and of 18 eggs treated with this process, only 3 contained surviving salmonellae (Table 3.2). Only this treatment was used for subsequent inoculation experiments.

![Figure 3.5 Untreated and heat-pasteurized eggs.](image)

Heat pasteurized eggs were cooled for two hours before breaking.
A: Untreated eggs  
B: Pasteurized at 54.4°C, 80 minutes (internal temp., holding time)  
C: Pasteurized at 56.1°C, 32 minutes (internal temp., holding time)  
D: Pasteurized at 57.7°C, 13 minutes (internal temp., holding time)

**Heat treatment profiles**

Profiles for heating of eggs to target internal temperatures are shown in Table 3.1. Once target temperature was achieved, internal temperatures continued to rise slightly throughout holding time. While this effect was observed in all treatments, deviation from target temperature was less than one degree in all cases.
Table 3.1 Summary of heating time and temperature changes of pasteurized eggs.

<table>
<thead>
<tr>
<th>Targeted treatment</th>
<th>Initial temp (°C)</th>
<th>Time to reach target (min)</th>
<th>Total heating time (min)</th>
<th>Final temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55°C for 20 min + ozone(^a)</td>
<td>27.6</td>
<td>24</td>
<td>44</td>
<td>55.9</td>
</tr>
<tr>
<td>56°C for 10 min + ozone(^a)</td>
<td>27.5</td>
<td>24</td>
<td>34</td>
<td>56.4</td>
</tr>
<tr>
<td>57°C for 5 min + ozone(^a)</td>
<td>25.3</td>
<td>25</td>
<td>30</td>
<td>57.4</td>
</tr>
<tr>
<td>58°C for 3 min + ozone(^a)</td>
<td>26.1</td>
<td>27</td>
<td>30</td>
<td>58.2</td>
</tr>
<tr>
<td>56.1°C for 32 min</td>
<td>27.7</td>
<td>21</td>
<td>53</td>
<td>56.4</td>
</tr>
</tbody>
</table>

Values represent the average of measurements taken from three eggs.
\(^a\)Ozone treatment comprised of vacuum (50.8 kPa), followed by ozone under pressure (maximum concentration of 160 g m\(^{-3}\), ~187.5 kPa) for 60 min.

Pasteurization with heat-ozone combination

Inoculated shell eggs were processed with heat (55-58°C) followed by an ozonation process (Table 3.2). Additionally, a commercially-relevant thermal pasteurization process (56.1°C for 32 min; Davidson, 2003) was tested as a reference treatment.

Table 3.2 Log reduction of *Salmonella Enteritidis* per gram of egg contents after treatment with heat or heat plus ozone.

<table>
<thead>
<tr>
<th>Treatment conditions(^a)</th>
<th>Average(^b) decrease in log CFU per g egg</th>
<th>Positive/total(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>56.1°C for 32 min</td>
<td>6.96 ± 0.38</td>
<td>3/18</td>
</tr>
<tr>
<td>55°C for 20 min plus ozone</td>
<td>7.14 ± 0.14</td>
<td>16/18</td>
</tr>
<tr>
<td>56°C for 10 min plus ozone</td>
<td>7.04 ± 0.13</td>
<td>10/18</td>
</tr>
<tr>
<td>57°C for 5 min plus ozone</td>
<td>6.82 ± 0.23</td>
<td>5/18</td>
</tr>
<tr>
<td>58°C for 3 min plus ozone</td>
<td>6.94 ± 0.12</td>
<td>1/18</td>
</tr>
</tbody>
</table>

\(^a\) Heat treatment time is holding time at target temperature indicated
\(^b\) Ozone treatment comprised of 50.8 kPa vacuum followed by maximum 160 g/m\(^3\) ozone at ~187.5 kPa for 60 min.
\(^c\) Averages for 36 treated eggs ± standard deviation

Presence of *Salmonella Enteritidis* confirmed by streaking of extended enrichment on xylose lysine desoxycholate agar.
All combination pasteurization treatments tested provided a large enough reduction in inoculated population (> 6-log CFU g⁻¹ egg contents in all instances) to be deemed “pasteurization.” There were no significant differences in inactivation among combination treatments, or between combination treatments and the selected heat-alone treatment, though the total number of eggs testing positive for *Salmonella* Enteritidis did vary according to treatment used (Figure 3.6).

![Figure 3.6 Percentage of pasteurized eggs testing positive for *Salmonella* Enteritidis. All processes (except the 56.1/32 min process) were followed with an ozone treatment. n=36 eggs per treatment.](image)

aEggs pasteurized by heat treatment displayed plus ozone treatment if applicable
bPositive result denoted by observation of *Salmonella*-typical colonies on XLD agar (incubated at 35°C for 24-48 hours) streaked with egg/peptone water homogenate previously incubated at 35°C for 48 hours.
cHeat treatment listed is target internal egg temperature and holding time at that temperature.
dOzone treatment applied following heat treatment (50.8 kPa vacuum followed by maximum 160 g/m³ ozone at ~187.5 kPa for 60 min)
eTreated by heating only
Though all treatments produced similar reductions in internal *Salmonella* Enteritidis populations, noticeable differences in visual acceptability were observed among combination pasteurized treatments. The general trend indicated that eggs heated to higher internal temperature displayed greater apparent albumen opacity. Because heating time decreased with increasing target temperature, these results suggest that internal temperature is more closely correlated with albumen denaturation than is heating time. Photographs of combination pasteurized eggs are displayed in Figure 3.7.
Figure 3.7 Eggs pasteurized with heat plus ozone combination treatments. All samples were subjected to heat treatment indicated followed by application of vacuum (50.8 kPa) followed by static treatment with ozone under pressure (maximum 160 g m\(^{-3}\), ~187.5 kPa, 60 minutes).
A: Heat treatment 55°C, 20 minutes
B: Heat treatment 56°C, 10 minutes
C: Heat treatment 57°C, 5 minutes
D: Heat treatment 58°C, 3 minutes

**Treatment with variable heating in combination with ozonation**

In order to verify that heating times used were necessary for corresponding lethality of overall treatment, experiments were conducted using a combination process comprised of heating to internal temperature of 56°C for reduced holding times (0, 3, or 5 minutes) without alteration of ozone treatment. The results of these experiments are
displayed in Figure 3.8. Inactivation of *Salmonella* Enteritidis in shell eggs was strongly affected by holding time of the heating step, with each decrease resulting in significantly larger surviving population.

**Figure 3.8 Reduction of *Salmonella* Enteritidis in eggs subjected to varying heat treatments followed by ozone treatment**

Error bars represent standard deviation (n=36)

*a* All treatments subjected to equivalent ozone treatment: application of vacuum (50.8 kPa) followed by static treatment with ozone under pressure (maximum 160 g m⁻³, ~187.5 kPa, 60 minutes)

**DISCUSSION**

The results of this study indicate that all treatments tested are sufficient to provide a pasteurized shell egg. Despite the indistinguishable reductions in internal contamination offered by all treatments, lethality, as defined by the total number of positive samples (Figure 3.6) seemed to vary slightly, with lower temperature, longer time treatments producing more eggs containing surviving *Salmonella* cells. This trend is in accordance with previous research conducted on *Salmonella* Enteritidis inactivation in
yolk (Grijspeerdt and Herman 2003). Though this information is useful for empirical comparison of treatments, the differences observed are not likely to be significant in natural vertically contaminated shell eggs. The high contamination levels of *Salmonella* used in this study were necessary to verify a 5-log reduction (or greater), but are several orders of magnitude greater than what would reasonably be expected in naturally contaminated eggs. Because naturally contaminated eggs most often contain very low levels of *Salmonella* Enteritidis (Humphrey et al. 1991), it is expected that all treatments used in this study would be capable of eliminating natural contaminants. When applied to an egg containing 100 cells of *Salmonella* Enteritidis, a process providing no less than 6-log reduction can be expected to result in pasteurized eggs containing no more than 0.000002 cells per egg, or one surviving cell per 500,000 contaminated eggs. If USDA estimates of internal contamination are accurate (1 in 20,000 eggs containing viable salmonellae), this would translate to only 1 in ten billion eggs containing viable salmonellae after application of treatment, barring process failure or other unpredictable confounding factors.

The importance of selecting treatment parameters carefully is clearly illustrated by the visual appearance of treated eggs (Figures 3.5 and 3.7). Though all treatments can be considered microbiologically equivalent, the time/temperature combinations used for heating steps are very close to the “critical points” for maintenance of egg quality previously alluded to by several researchers (Hou et al. 1996, Schuman 1996, Rodriguez-Romo 2004, van Lith et al. 1995). Attempts to reduce heating time without increasing treatment temperature were made, but this resulted in unacceptable decrease in lethality of *Salmonella* Enteritidis (Figure 3.8), when compared to original holding time.
Thermally pasteurized shell eggs currently available in the retail market provide an unchallenged record of safety. However, they still comprise only a small portion of the total shell egg market, accounting for only 5% of table eggs produced in the United States (United States Department of Agriculture, Food Safety and Inspection Service 2005). This may be partly due to reduced consumer acceptance on the basis of altered appearance (albumen opacity) and/or reduced functionality (increased whipping times). Providing a pasteurized product to a larger segment of the consumer base by mitigating these problems is an important step toward achieving the goals for reduction in *Salmonella* Enteritidis illnesses set forth in both the President’s Council on Food Safety egg action plan (1999) and the Healthy People 2010 report (President’s Council on Food Safety, United States Department of Health and Human Services 2009).

The current study verifies that a pasteurized shell egg of comparable safety can be produced with reduced overall heating time using a combination of immersion heating plus ozone. As discussed previously (Perry et al. 2008), the application of two technologies is likely to confer an additional degree of safety by providing a means to inactivate the small (often uncountable) portion of cells responsible for the tailing effect commonly observed in food systems (resistant sub-populations), which may not be eliminated even after extended heating. Furthermore, because the proposed heat-ozone combination process requires less overall heating time, it is likely to reduce the degree of protein denaturation in the resulting egg, meaning that egg functionality will be less severely affected when compared to a product produced using a comparable thermal-only process. Increase in opacity, a marker which has previously been used as a designation of protein denaturation (Hou et al. 1996, Rodriguez-Romo 2004, Schuman et al. 1997),
of eggs pasteurized using this process can be greatly mitigated by the modification of treatment parameters, without compromising safety (Table 3.1 and Fig. 3.6).

In a visual-only sensory study conducted at The Ohio State University on eggs processed by a procedure similar to that used in this study, consumer participants rated both the albumen and yolk of thermally pasteurized eggs (Davidson’s Safest Choice brand) as significantly cloudier compared to ozone treated eggs (Kamotani 2010). This finding suggests that ozone processed eggs may enjoy better acceptance than current pasteurized shell eggs, if introduced into the marketplace. *Salmonella* contamination can be introduced to laying flocks through contaminated feed, contact with rodents and insects, or even soil (Davies and Breslin 2003). Cross contamination can occur even during washing (Musgrove et al. 2005). Though improvements in sanitation throughout the production chain have made progress toward mitigating these concerns, pasteurization provides an unparalleled level of safety. Technology that can be used to expand availability and consumer choice in this market would be strongly beneficial. The data presented above indicates that heat-ozone combination processing is a strong candidate to meet this need. Despite the progress achieved with this study, there remains a need for more direct study of the quality parameters and functional performance of ozone-pasteurized eggs in order to verify the maintenance of their quality throughout the normal shelf-life of shell eggs.
REFERENCES


President's Council on Food Safety. 1999. Egg safety from production to consumption: an action plan to eliminate Salmonella Enteritidis illnesses due to eggs.


Chapter 4
Quality and functionality of shell eggs pasteurized with a heat and ozone combination process

ABSTRACT

Recently, an ozone-heat process has been designed and tested against Salmonella enterica serovar Enteritidis in shell eggs. While microbiologically efficacious, the quality of eggs treated with this process has not been verified. The purpose of this study is to assess the physical quality and functionality of shell eggs that have been pasteurized with a combination of heat and ozone. Shell eggs were pasteurized by one of two methods: i) immersion heating to a targeted internal temperature of 56°C for 32 min, or ii) heat (targeting 56°C for 10 min) followed by ozone treatment. The ozonation process involved application of vacuum (50.8 kPa) followed by static ozone treatment (160 g m⁻³ maximum concentration at ~187.5 kPa for 60 min). Immediately after treatment, eggs were stored at 4 or 25°C, and analyzed biweekly for up to eight weeks. Samples were tested for Haugh units, albumen pH, albumen turbidity, yolk index and percent overrun.

Processed and unprocessed eggs maintained superior quality when stored at 4°C as opposed to 25°C. Pasteurization, regardless the method, maintained egg quality markers during storage, with few exceptions. Haugh values were maintained better
during storage in both pasteurized samples, when compared to untreated eggs. Turbidity of albumen increased in this order: untreated, combination-treated, heat-treated shell eggs. Pasteurization significantly decreased overrun; this decrease in albumen functionality was less severe in combination-treated than in heat-treated eggs. Albumen pH was significantly lower in combination-treated eggs, compared to other treatments. In conclusion, when microbiologically equivalent heat and combination processes are employed, eggs treated with the combination process have an advantage in terms of quality.

INTRODUCTION

Foodborne illness caused by *Salmonella enterica* serovar Enteritidis (*Salmonella Enteritidis*) is a serious health concern that has received a great deal of attention in recent decades (Gantois et al. 2009, St Louis et al. 1988, Braden 2006, Guard-Petter 2001). It is estimated that contaminated eggs result in almost 200,000 illnesses annually in the United States alone (Schroeder et al. 2005). Attempts to mitigate the severity of this problem have been numerous. These include regulation requiring all breeding stock to be certified free of *Salmonella* Enteritidis, increased traceback efforts to identify infected laying flocks, institution of egg quality assurance programs focusing on environmental testing and safe egg handling, and consumer education (Hogue et al. 1997). Most recently, a final rule was passed in July 2009 requiring testing of both flocks and laying environments with provisions for uses of eggs from contaminated flocks (United States Food and Drug Administration 2009). This rule also requires prompt refrigeration of
eggs after laying, an important step in preventing bacterial migration and multiplication within egg contents.

In a plan released in 1999 by the President’s Council on Food Safety, the addition of a bactericidal processing step to conventional egg processing was recommended as a measure of primary importance to reduce egg-associated illness (President's Council on Food Safety 1999). A 1998 risk assessment conducted by the United States Department of Agriculture’s Food Safety and Inspection Service estimated that the implementation of a mandatory processing step resulting in at least 3 log reduction of *Salmonella* Enteritidis in shell eggs would drastically decrease illnesses (United States Department of Agriculture, Food Safety and Inspection Service 2005).

Shell eggs pasteurized by immersion in heated water are currently available in the marketplace. However, the process that is used to treat these eggs consists of extended heat treatment, which has been reported to produce undesirable changes to the quality and functionality of the resulting egg (Rodriguez-Romo 2004, Schuman 1996, Dev et al. 2008). In 2005, pasteurized shell eggs accounted for only 5% of the total market (United States Department of Agriculture, Food Safety and Inspection Service 2005). Reduced quality could be a primary reason for the lack of large-scale consumer acceptance of pasteurized shell eggs.

Egg quality can be measured in a number of ways and each portion of the egg is addressed separately. The most common method for assessment of shell quality is candling. This allows visualization of hairline cracks in the shell and measurement of air cell size. Shell strength is sometimes tested by measuring the force required to break the intact shell. The most common measurement of albumen quality is the Haugh unit. This
index, developed in 1937, expresses the relationship between the height of the thick albumen and the weight of the whole egg as a single, unitless value (Haugh 1937). Higher values denote better quality, and minimum required Haugh unit values are specified in the guidelines for egg grading (United States Department of Agriculture, Agricultural Marketing Service 2000). The most accepted measurement of yolk quality is the yolk index, a ratio of the height to width of the egg yolk. Vitelline membrane strength is sometimes measured in a manner similar to that used for measurement of shell strength. The changes in these measurements over prolonged storage have been previously investigated (Jones and Musgrove 2005). This study employs several tests to compare the quality of pasteurized eggs (using heat only and combination processes) to that of untreated eggs during extended storage.

MATERIALS AND METHODS

Eggs

Unfertilized, washed Grade AA large eggs were obtained from the farms of Hemmelgarn & Sons, Inc. (Coldwater, OH). Eggs were stored at 4°C until treatment and were treated within two weeks of laying. All eggs were weighed and only those weighing 2 oz ± 5% (56.7 ± 2.8 g) were used. Selected eggs were stored at room temperature for approximately 18 hours before treatment. Untreated eggs were left at room temperature until pasteurization treatments were finished, at which time all eggs were stored in formed pulp trays at either 4°C or 25°C until analysis.
Heat Pasteurization

Eggs were placed on a custom-made stainless steel rack and heat-treated by immersion in a circulating water bath (Thermo Corporation, Waltham, MA) set to 57°C. Temperature in the approximate center of the yolk was monitored using an un-inoculated egg with thermocouple (Fluke, Everett, WA) wire inserted to the approximate center of the yolk. Once eggs reached target internal temperature (56.1°C), they were held in the water bath for an additional 32 minutes. Internal temperature increased slightly during holding time (Figure 4.2). Immediately after treatment, eggs were transferred into formed pulp trays for storage at either 4°C or 25°C until analysis.

Heat plus Ozone (Combination) Pasteurization

Eggs were placed onto a custom-made stainless steel rack and heat treated by immersion in a circulating water bath (Thermo Corporation, Waltham, MA) set to 57°C. Temperature in the approximate center of the yolk was monitored using an un-inoculated egg with thermocouple (Fluke, Everett, WA) wire inserted to the approximate center of the yolk. Once eggs reached target internal temperature (56°C), they were held in the water bath for an additional 10 minutes. Internal temperature during heating is displayed in Figure 4.2. Immediately after heat treatment, the rack was transferred to a custom-made stainless steel vessel for ozone treatment. Eggs were subjected to vacuum (50.8 kPa) prior to ozone treatment. Gaseous ozone was produced from pure oxygen by an ozone generator (Ozonia, Elmwood Park, NJ). Ozone gas was pumped to a custom treatment vessel to reach a maximum ozone concentration of 160 g m\(^{-3}\). Ozone concentration was monitored continually using an ultraviolet ozone detector (Mini-Hicon
model; IN USA, Inc., Norwood, MA). Introduction of ozone caused increase in pressure, with the final pressure being ~187.5 kPa. Once desired ozone concentration and pressure were reached (come-up time of 10 - 12 minutes), gas inlet was closed and eggs were subjected to static treatment for 60 minutes. At the end of treatment, pressure and ozone were released from the vessel and ozone was destroyed by a thermal destruct unit (Ozonia, Elmwood Park, NJ). Residual ozone was flushed from the vessel using compressed air. Treated eggs were removed from the vessel and placed in formed pulp trays for storage at 4°C or 25°C until analysis.

**Quality testing**

Eggs were tested for quality factors immediately after treatment (Day 0) and biweekly thereafter. Three eggs from each storage temperature/treatment combination were tested on each testing date. Eggs stored at 4°C were allowed to warm at room temperature for at least three hours prior to testing to avoid artificially high Haugh unit and yolk index values. The entire experiment was performed in duplicate. Eggs were analyzed for the following indices.

**Yolk index**

Eggs were broken onto a level surface. The width and height of egg yolk was measured using Vernier calipers (Manostat Co., Merenschwand, Switzerland). Values were recorded and used to calculate yolk index as follows:

{\[
\text{Yolk height/Yolk width} = \text{Yolk index}
\]}
**Haugh units**

Eggs were cracked into a plastic dish resting on a level surface. Haugh units were measured using a Haugh unit meter (Mattox & Moore, Indianapolis, IN). Egg weight is set manually on the meter before measurements are taken, the resulting dial reading equals Haugh units for the egg tested. The formula for Haugh units is:

\[ HU = 100 \log(h-.01*5.6745(30w^{37}-100)+1.9) \]

Where HU = Haugh units, h = thick albumen height and w = weight of the unbroken egg. The meter was placed over the egg with measuring pin positioned approximately half way between the outer edge of the yolk and outer edge of thick albumen, avoiding the chalzae. Measurements were taken in three different areas of the albumen and averaged.

**Albumen pH**

Albumen was separated from the yolk using the egg shell. It was placed in a glass beaker and stirred on a magnetic stirrer (Thermix Model 210T, Fisher Scientific, Pittsburg, PA) at low speed for one minute. pH was measured using a standard pH meter (Accumet model 15, Fisher Scientific, Pittsburgh, PA).

**Albumen turbidity**

Albumen was separated from the yolk and stirred. Two ml of homogenized albumen was pipetted into disposable cuvettes. Absorbance was measured at 600 nm in a spectrophotometer (Spectronic 20 Genesys, Spectronic Instruments, Leeds, UK). Samples were read against a blank of distilled water.
**Albumen overrun**

Albumen was separated from yolk, placed in an adjustable measuring cup (Adjust-a-cup, KitchenArt, Florence, AL) (Figure 4.1), and tared. Tared weight was recorded. Albumen was whipped using a hand mixer (Chefmate, St. Louis MO) on high speed for ten minutes. At specified intervals (1, 2, 3, 4, 5, and 10 minutes), samples of foam were taken (equal to original volume of unwhipped albumen) and tared weight was recorded. Percent overrun at each of six time points was calculated using the formula below:

\[
\text{% Overrun} = 100 \times \frac{[\text{wt. original volume egg white}) - (\text{wt. original volume foam})]}{(\text{wt. original volume egg foam})}
\]

![Figure 4.1 Adjustable measuring cup used to determine weight of specific volume of egg white foam.](http://www.kitchenart.com/detail.aspx?ID=247)
Statistical Analysis

Data analysis was performed using SAS v. 9.1.3 software (SAS Institute, Inc., Cary, NC). Analysis was performed separately for each quality parameter using the MIXED procedure. The following model was used with treatment, storage temperature and storage time identified as class variables:

Parameter value = \mu + \text{treatment} + \text{storage temperature} + \text{storage time} +
(treatment*storage time) + (treatment*storage temperature) + (storage temperature*storage time) + (treatment*storage temperature*storage time)

Significant differences were identified using difference of least squares means comparisons with \( p < 0.05 \) considered significant.

RESULTS

The heating profile of eggs exposed to heat only and combination treatments is displayed in Figure 4.2. Heating profile and time taken to reach target temperature were similar between treatments, with the only major difference being the extended heating used for heat only pasteurization.
Yolk index

Yolk index measurements for all treatments stored at 4°C are displayed in Figure 4.3. Untreated and combination pasteurized eggs did not display a significant change in yolk index over the storage period. The yolk index of heat pasteurized eggs decreased significantly ($p < 0.05$) from week two to four and from week six to eight, indicating decreased strength of the vitelline membrane. After eight weeks of storage at 4°C, yolk index was not significantly different among treatments.
Figure 4.3 Yolk indices of untreated or pasteurized\textsuperscript{a} shell eggs during eight weeks of storage at 4°C.

Error bars indicate standard error (n=36)

\textsuperscript{a}Eggs pasteurized by heat only (56.1°C internal egg temperature for 32 min) or heat/ozone combination process (56°C internal egg temperature for 10 min followed by 15 inHg vacuum followed by maximum 160 g/m\textsuperscript{3} ozone at \textasciitilde 187.5 kPa for 60 min) as indicated

*Indicates significant change ($p < 0.05$) in yolk index compared to previous testing date within treatment

Yolk index measurements for all treatments stored at 25°C are displayed in Figure 4.4. Decreases in measurements over time were more pronounced at 25°C than at 4°C (as expected). Untreated eggs displayed the most dramatic decrease in yolk index, with the decrease becoming significant at the two week mark. Significant decrease in heat-pasteurized eggs was observed starting at week four, while yolk index of combination pasteurized eggs did not decrease significantly until the last testing date (week 8). These data indicate superior maintenance of yolk quality in combination pasteurized samples.
While all three treatments displayed similar yolk index values on day zero, the values in untreated eggs decreased significantly more rapidly during storage than those in eggs treated with either pasteurization process, which were statistically indistinguishable throughout the storage period.

Figure 4.4 Yolk indices of untreated or pasteurized\(^a\) shell eggs during eight weeks of storage at 25°C.

Error bars indicate standard error (n=6)

\(^a\)Eggs pasteurized by heat only (56.1°C internal egg temperature for 32 min) or heat/ozone combination process (56°C internal egg temperature for 10 min followed by 15 inHg vacuum followed by maximum 160 g/m\(^2\) ozone at ~187.5 kPa for 60 min) as indicated

*Indicates significant change (\(p < 0.05\)) in yolk index compared to previous testing date within treatment
Additionally, a significant storage temperature effect could be observed in untreated eggs. Untreated eggs stored at 25°C displayed significantly lower values for yolk index at every testing date, after day zero, than did untreated eggs stored at 4°C. This effect was less severe in heat pasteurized eggs; there was no significant difference between eggs stored at different temperatures until weeks six and eight. In combination pasteurized eggs, a significant decrease was observed only at week eight.

**Haugh units**

The change in Haugh unit measurements in eggs stored at 4°C over eight weeks of storage is displayed in Figure 4.5. No significant differences within treatment were observed from day zero to week eight. There were no differences observed between the pasteurized treatments, while untreated samples consistently yielded significantly lower values than treated eggs.
Differences in Haugh units among samples stored at 25°C were slightly more pronounced, and are displayed in Figure 4.6. Neither heat pasteurized nor combination pasteurized samples displayed a significant change from week to week, but measurements in untreated samples decreased significantly throughout the first four weeks of storage, as expected. No significant differences between heat and combination pasteurized samples was observed, while Haugh units of untreated eggs were significantly lower than those subjected to either pasteurization treatment.
Figure 4.6 Haugh units of untreated and pasteurized shell eggs during eight weeks of storage at 25°C.
Error bars indicate standard error (n=12)

*Eggs pasteurized by heat only (56.1°C internal egg temperature for 32 min) or heat/ozone combination process (56°C internal egg temperature for 10 min followed by 15 inHg vacuum followed by maximum 160 g/m³ ozone at ~187.5 kPa for 60 min) as indicated

*Indicates significant change ($p < 0.05$) in Haugh units compared to day 0 within treatment

**Albumen pH**

The change in albumen pH in eggs stored at 4°C over eight weeks of storage is displayed in Figure 4.7. No significant differences within treatment were observed from day zero to week 8 in untreated eggs. In heat pasteurized eggs, there was a significant increase in pH from day zero to week two. In the same time period, combination pasteurized samples displayed a significant decrease, which was maintained throughout the storage period. Throughout storage, untreated and heat pasteurized eggs were indistinguishable, while combination pasteurized eggs maintained a significantly lower...
pH. This lower pH is a positive attribute from a quality standpoint, as pH increases due to loss of CO₂ is positively correlated with egg age (Romanoff and Romanoff 1949).

Figure 4.7 Albumen pH of untreated and pasteurized shell eggs during eight weeks of storage at 4°C.
Error bars indicate standard deviation (n=12)
*Eggs pasteurized by heat only (56.1°C internal egg temperature for 32 min) or heat/ozone combination process (56°C internal egg temperature for 10 min followed by 15 inHg vacuum followed by maximum 160 g/m³ ozone at ~187.5 kPa for 60 min) as indicated
*Indicates significant change (p < 0.05) in albumen pH compared to day 0 within treatment.

The change in albumen pH measurements in eggs stored at 25°C over eight weeks of storage is displayed in Figure 4.8. In heat pasteurized and untreated eggs, there was a significant increase in pH from day zero to week two. In the same time period,
combination pasteurized samples displayed a significant decrease, which was maintained throughout the storage period. These results are very similar to those observed in eggs stored at 4°C with the exception of higher final albumen pH (9.3 as opposed to 9.1) in untreated and heat pasteurized samples. The pH of combination-pasteurized samples was similar at both temperatures.

Figure 4.8 Albumen pH of untreated and pasteurized shell eggs during eight weeks of storage at 25°C.
Error bars indicate standard deviation (n=12)
*Eggs pasteurized by heat only (56.1°C internal egg temperature for 32 min) or heat/ozone combination process (56°C internal egg temperature for 10 min followed by 15 inHg vacuum followed by maximum 160 g/m³ ozone at ~187.5 kPa for 60 min) as indicated
*Indicates significant change (p < 0.05) in albumen pH compared to day 0 within treatment
**Albumen turbidity**

The change in albumen turbidity (measured as absorbance at 600 nm) in eggs stored at 4°C over eight weeks of storage is displayed in Figure 4.9. No significant differences were observed week-to-week within treatment. Absorbance for untreated eggs remained relatively unchanged over the course of the study, but some fluctuation could be observed in pasteurized samples; however, these changes were not significant. The same trends were observed in samples stored at 25°C (data not shown). On every testing date, including day zero, albumen turbidity was significantly different among treatments, increasing from untreated eggs to combination pasteurized eggs, to heat pasteurized eggs. This suggests that while the heat treatment employed in the combination pasteurization process is damaging to albumen, it is significantly less damaging than a heat alone pasteurization treatment. Eggs held at 25°C displayed the same trends (data not shown).
Figure 4.9 Albumen turbidity of untreated and pasteurized shell eggs during eight weeks of storage at 4°C.
Error bars indicate standard error (n=12)

*Eggs pasteurized by heat only (56.1°C internal egg temperature for 32 min) or heat/ozone combination process (56°C internal egg temperature for 10 min followed by 15 inHg vacuum followed by maximum 160 g/m³ ozone at ~187.5 kPa for 60 min) as indicated

**Overrun**

Albumen overrun was measured after 1-10 minutes of whipping. Percent overrun over whipping time (for all samples) on Day 0 is displayed in Figure 4.10. This figure demonstrates that final overrun values are drastically different among samples, with untreated eggs yielding much greater increase in volume. Eggs pasteurized by either treatment also required longer whipping time to reach their final overrun values.
Figure 4.10 Percent overrun of albumen from untreated and freshly pasteurized shell eggs during 10 minutes of whipping.
Error bars indicate standard error (n=12)

Storage of eggs at 4°C for eight weeks did not affect the overrun values achieved after ten minutes of whipping (Figure 4.11), but storage did lead to longer whipping times required to reach final volume, as evidenced by the more linear shape of the curves.
Figure 4.11 Percent overrun of albumen from untreated and pasteurized shell eggs stored for 8 weeks at 4°C during 10 minutes of whipping. Error bars indicate standard error (n=12).

*Eggs pasteurized by heat only (56.1°C internal egg temperature for 32 min) or heat/ozone combination process (56°C internal egg temperature for 10 min followed by 15 inHg vacuum followed by maximum 160 g/m³ ozone at ~187.5 kPa for 60 min) as indicated.

Eight weeks of storage at 25°C did not alter overrun values achieved after ten minutes in untreated and combination-pasteurized eggs, but final overrun value decreased significantly in heat-pasteurized eggs (Figure 4.12), which produced an average of only less than 100% overrun on the final testing date, less than one quarter of the value produced by heat-pasteurized eggs stored at 4°C.
At all time points, overrun in untreated eggs was significantly greater than that of combination pasteurized eggs, which was significantly higher than values obtained from heat treated eggs from weeks 4-8 (data not shown). An effect due to storage temperature could be observed only in heat pasteurized samples, in which percent overrun decreased significantly more over the storage period at 25°C, compared to 4°C.

**DISCUSSION**

Trends observed for yolk index and Haugh unit measurements were similar. Though few differences could be observed when eggs were stored at 4°C, storage at 25°C resulted in significant decrease in both quality markers in untreated eggs only. Both
pasteurized treatments performed better under temperature abuse conditions than did untreated eggs. This can be attributed to unavoidable thermal denaturation resulting from the heat treatment used in both processes. As eggs age, water migrates from the albumen to the yolk and the strength of the vitelline membrane decreases (Romanoff and Romanoff 1949). The combination of these two factors allows the yolk to spread, reducing its height and consequentially yolk index. The application of heat serves to partially denature the proteins comprising this membrane, which causes a maintenance of rigidity that otherwise would not be observed. Similarly, Haugh unit values are known to decrease during storage (Scott and Silversidest 2000). The cause of this decrease has not been well explained, but is believed to be due to the dissociation of the ovomucin-lysozyme complex as albumen pH values increase, and a negative correlation between albumen height and pH has been demonstrated (Scott and Silversidest 2000), supporting this theory. Partial denaturation of albumen proteins leads to aggregation, which increases the height of the thick albumen and accordingly, Haugh unit values. The increase in Haugh units due to protein denaturation is recognized as a contradiction in terms, but this measurement remains the predominant assessment used to quantify egg quality. USDA requirements state that Grade AA eggs must measure a minimum of 70 Haugh units (United States Department of Agriculture, Agricultural Marketing Service 2000), with no provisions for pasteurized eggs. Using this standard, all samples except untreated eggs stored at 25°C maintained Grade AA quality for the duration of this study.

Increase in albumen pH as eggs age has been well documented (Scott and Silversidest 2000, Chen et al. 2005). It is attributed to the loss of free carbon dioxide associated with the albumen. The pH of albumen in a newly laid egg is expected to be in
the range of 7.6, but that value increases to almost 9 after less than two weeks of storage (Powrie and Nakai 1986). Oiling of shell eggs, which reduces gas exchange through the shell, has been shown to prevent a rise in albumen pH (Sabrani and Payne 1978). This trend was observed as expected in untreated and heat pasteurized eggs (Figures 4.7 and 4.8), but the application of ozone seemed to prevent drastic changes in albumen pH regardless of subsequent storage temperature. This result was also observed in previous tests using ozone treated eggs (Chapter 2). Because CO₂ is expected to act as a radical scavenger, and ozone decomposition gives rise to several radical species, the application of ozone would be logically expected to further decrease levels of CO₂ in the albumen. This, however, would produce an effect exactly opposite of that observed. It is expected that the sustained lower pH is the result of radical chain reactions on some component of albumen, but further investigation would be necessary to identify specific possibilities to explain this outcome.

A clear trend was observed in albumen turbidity measurements. As expected, the application of heat led to a noticeable increase in albumen turbidity; this is in accordance with previous results obtained by other researchers (Rodriguez-Romo 2004, Schuman et al. 1997). However, in this study, the direct benefit of the reduced heating utilized in combination treatments can be detected. While combination-pasteurized eggs were significantly more turbid than untreated eggs at all testing dates regardless of storage temperature, they were also significantly less turbid than heat-pasteurized eggs. This result strongly indicates a reduced amount of protein denaturation in the albumen of combination pasteurized eggs when compared to eggs treated with heat alone.
Interpretation of overrun data can be approached in two ways; i). the maximum level of overrun achieved, and ii). the amount of whipping needed to achieve high levels of overrun. After ten minutes of whipping, overrun was significantly higher in untreated samples. When stored at 4°C, heat pasteurized eggs yielded significantly lower overrun percentages on multiple testing dates, compared to combination-pasteurized samples. When stored at 25°C, combination treated eggs performed significantly better than heat pasteurized eggs, but not as well as untreated eggs. This trend reinforces the information gained from albumen turbidity measurements, that while combination pasteurization does cause some damage to egg components, it is significantly less damaging than pasteurization with heat alone.

The differences among treatments are much clearer when a second approach is used to assess these data. While untreated eggs attain nearly half of their maximum volume after only one minute of whipping, the increase in overrun is much more gradual in pasteurized samples (Figures 4.10 – 4.12). This indicates damage to one or more of the proteins involved in albumen foaming. Foaming ability is attributed to a combination of globular proteins (which enhance volume) and ovomucin (which provides stability) (Romanoff and Romanoff 1949). Damage to either of these proteins is likely to impair whipping, as observed in pasteurized samples. Globular proteins G₂ and G₃ are considered to be some of the more heat labile albumen proteins, while ovomucin is considered to be exceptionally stable, withstanding heat treatment at 90°C (Powrie and Nakai 1986). While this would suggest that globulin denaturation is responsible for the observed decrease in foaming ability, ovomucin is often found in albumen to be complexed with lysozyme, which because of its alkaline isoelectric point, is very seldom
found apart from either ovomucin or ovalbumin. Though lysozyme is considered to be fairly heat stable, with a denaturation temperature of over 70°C at pH 7, its susceptibility to heat increases with increasing pH. Within the environment of the egg (pH ~9), lysozyme can be expected to denature at temperatures closer to 60°C (Powrie and Nakai 1986). Additionally, work conducted on ovomucin-lysozyme complexes suggests that the aggregate may be significantly more susceptible to denaturation by heat than either of the individual component proteins (Garibaldi et al. 1968). Without further testing, the specific protein most affected by heat treatments cannot be identified.

Taken as a whole, the results of this study can lead to several conclusions regarding the physical quality of eggs pasteurized by application of heat and gaseous ozone. This treatment seems to render eggs much less susceptible to the quality deterioration that is expected in untreated eggs over time. Additionally, exposure of treated eggs to temperature abuse does not compromise egg quality as measured in this study. While the functionality of egg albumen is decreased by combination pasteurization, this decrease is slightly less significant than that seen in eggs pasteurized by other methods, and we propose, is compensated for by the increased safety assurance provided by this novel treatment.

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Chapter 5

Changes in Albumen Proteins Due to Pasteurization of Whole Eggs with Heat or Heat Ozone Combinations

ABSTRACT

Pasteurization of shell eggs effectively reduces internal contamination with *Salmonella* Enteritidis by a minimum of 5 log CFU g⁻¹. Previous research on pasteurization of shell eggs with heat-alone and heat-ozone combination has verified the microbial lethality of these processes and assessed the physical quality of the resulting products. Physical tests on albumen of treated eggs have led to the conclusion that both heat-only and heat-ozone combination processes damage albumen to some degree. In this study, treated and untreated eggs were subjected to a number of analyses to further characterize the changes resulting from egg pasteurization by two distinct procedures. Eggs were assayed for lysozyme activity and free sulfhydryl group content. They were also analyzed using differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR). Results indicate that neither heat-alone nor combination pasteurization decreased lysozyme activity; in fact, activity was slightly greater in treated eggs. Differences in sulfhydryl group content among treatments were not detected. According to DSC thermograms, ovotransferrin was not damaged by either pasteurization treatment. A conversion of ovalbumin to S-ovalbumen with increasing heat treatment is readily apparent. FTIR analysis suggested slight differences in protein secondary
structure, indicating that $\alpha$-helix structure was lost in favor of $\beta$-sheets as heating time increased. While pasteurization of shell eggs by heat or heat plus ozone does not cause severe changes in protein structures, some effects were noticeable in eggs treated with heat alone. Consequently, heat-ozone pasteurization, by virtue of its less severe heat treatment, yields a safe final product that more closely resembles untreated shell eggs.

**INTRODUCTION**

Substantial research has been conducted regarding treatments that may be applied to shell eggs to reduce the risk of internal *Salmonella* contamination. The use of gamma radiation at levels of up to 3 kGy is an approved process for pasteurization of shell eggs (United States Food and Drug Administration 2000). Heat-based processes are used to produce two brands of pasteurized egg currently available in the marketplace. Research in this laboratory has led to the development of a process combining heat and gaseous ozone treatments to inactivate greater than 6 log CFU/g of *Salmonella Enteritidis* that has been grown in the yolk of intact eggs. Although these treatments increase the safety of shell eggs, some may damage egg albumen. In the case of gamma irradiation, the damage is severe enough to have prevented the implementation of this technology on a commercial scale. While the damage resulting from heat pasteurization is not considerable enough to preclude the use of the process, resulting changes can be visually detected by consumers, leading to decreased acceptance of the final product (Kamotani 2009).

Egg albumen is comprised of 88% water by weight (Romanoff and Romanoff 1949). The remaining portion is comprised overwhelmingly of protein. The number of
individual proteins contained in egg albumen has yet to be definitively determined, though use of SDS-PAGE to separate albumen components has detected up to 22 distinct bands (Desert et al. 2001). The most abundant albumen protein is ovalbumen, a glycoprotein with two known conformations, stressed and relaxed (Stevens 1991). The relaxed (or R) form is the more abundant of the two; though conversion to the S form, which is more stable to heat, is known to increase with age or application of heat (Stevens 1991, Rodriguez-Romo 2004). Ovomucin is responsible for whipping performance as well as the increased viscosity of the thick portion of the albumen, where it is more abundant (Kato et al. 1985). Ovomucoid, a serine proteinase inhibitor; lysozyme, an antimicrobial; ovotransferrin, also known as ovoconalbumen, an iron binding protein, and globular proteins of unknown function are the other prevalent proteins (Romanoff and Romanoff 1949).

The heat treatments used for commercial egg pasteurization are relatively mild. Eggs are generally heated at $\leq 58^\circ$C in order to prevent the most egregious adverse effects. At these temperatures, none of the major albumen proteins is expected to denature. Regardless, previous tests on opacity of albumen and whipping performance confirm that heat pasteurization results in damage to albumen (Chapter 4). Previous measurements of physical quality have indicated that albumen damage in eggs treated with the ozone combination process is less severe than that resulting from heat alone pasteurization, but information regarding the effect of these treatments on specific albumen proteins is lacking. This work combines several techniques in an attempt to ascertain what changes result from heat alone and heat-ozone pasteurization treatments.
MATERIALS AND METHODS

Eggs

Unfertilized, washed Grade AA large eggs were obtained from the farms of Hemmelgarn & Sons, Inc. (Coldwater, OH). Eggs were stored at 4°C until treatment and were treated within two weeks of laying. Eggs were equilibrated to 25°C by immersion in a water bath set to 25°C before treatment. Untreated eggs were left at room temperature until pasteurization treatments were finished, at which time all eggs were stored in formed pulp trays at 4°C until analysis.

Heat Pasteurization

Eggs were placed on a custom-made stainless steel rack and heat treated by immersion in a circulating water bath (Thermo Corporation, Waltham, MA) set to 57°C. Temperature in the approximate center of the yolk was monitored using an un-inoculated egg with thermocouple (Fluke, Everett, WA) wire inserted to the approximate center of the yolk. Once eggs reached target internal temperature (56.1°C), they were held in the water bath for 32 minutes. Immediately after treatment, eggs were transferred into formed pulp trays for storage at 4°C until analysis.

Heat plus Ozone (Combination) Pasteurization

Eggs were placed on a custom-made stainless steel rack and heat-treated by immersion in a circulating water bath (Thermo Corporation, Waltham, MA) set to 57°C. Temperature in the approximate center of the yolk was monitored using an un-inoculated egg with thermocouple (Fluke, Everett, WA) wire inserted to the approximate center of
the yolk. Once the eggs’ target internal temperature (56°C) was reached, eggs were held in the water bath for 10 minutes. Immediately after heat treatment, the rack was transferred to a custom-made stainless steel vessel for ozone treatment. Eggs were subjected to vacuum (50.8 kPa) prior to ozone treatment. Gaseous ozone was produced from pure oxygen by an ozone generator (Ozonia, Elmwood Park, NJ). Ozone gas was pumped to a custom treatment vessel to reach a maximum ozone concentration of 160 g m⁻³. Ozone concentration was monitored continually using an ultraviolet ozone detector (Mini-Hicon model; IN USA, Inc., Norwood, MA). Introduction of ozone caused increase in pressure, with the final pressure being ≤187.5 kPa. Once desired ozone concentration and pressure were reached (come-up time of 10 - 12 minutes), gas inlet was closed and eggs were subjected to static treatment for 60 minutes. At the end of treatment, pressure and ozone were released from the vessel and ozone was destroyed by a thermal destruct unit (Ozonia, Elmwood Park, NJ). Residual ozone was flushed from the vessel using compressed air. Treated eggs were removed from the vessel and placed in formed pulp trays for storage at 4°C until analysis.

**Determination of Sulfhydryl Groups and Disulfide Bonds**

Sulfhydryl groups were quantified using the method of Beveridge, et al. (Beveridge et al. 1974). Briefly, sulfhydryl groups are determined directly via a colorimetric reaction induced by Ellman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid)). The disulfide bond in Ellman’s reagent is cleaved by reaction with a protein thiol group in a stoichiometric fashion as displayed in Figure 5.1, producing a yellow color that is read at a wavelength of 412 nm. Reduction of protein samples with a combination of
urea and mercaptoethanol allows indirect quantitation of disulfide bonds. Color was developed for ten minutes before reading samples at 412 nm. Translation of spectrophotometric readings to concentrations was accomplished using the following equation.

$$\text{µM SH/g} = \frac{(73.53 \text{Abs}_{412} \times \text{dilution factor})}{\text{sample solids (mg/ml)}}$$

The dilution factor for the unreduced sample was 30.2, and for the reduced sample, it was 150. Sample solids were 120 mg/ml egg albumen. Fifteen eggs from each treatment were analyzed.

![Reaction of Ellman's reagent with thiol group to produce colored product.](image)

**Figure 5.1 Reaction of Ellman's reagent with thiol group to produce colored product.**

**Determination of Lysozyme Activity**

Lysozyme activity was assayed using the EnzChek Lysozyme Assay Kit (Invitrogen, Carlsbad, CA). This kit is based on interaction of lysozyme with *Micrococcus lysodeikticus* cell wall material that has been labeled with fluorescein. Interaction of lysozyme with cell wall material separates the dye from a built in quencher, allowing it to fluoresce. Samples of thin albumen (50 µl) were combined with the kit substrate containing cell wall material and incubated at 37°C for 60 minutes, at which time, fluorescence was read using a Bio-Rad qPCR thermocycler (Bio-Rad, Hercules,
CA). Samples were run alongside an eight point standard curve (Figure 5.2) prepared with purified lysozyme which was used to convert fluorescence readings to lysozyme concentrations. Fifteen eggs from each treatment were analyzed in duplicate.

![Figure 5.2 Lysozyme standard curve](image)

Error bars indicate standard deviation (n=3).

**Differential Scanning Calorimetry (DSC)**

DSC analysis of protein denaturation temperature was conducted using a modulated differential scanning calorimeter (Model 2920, TA Instruments, New Castle, DE). Thin albumen was pooled from five eggs in each treatment group. An aliquot of this sample (~35 mg) was placed in a high-volume stainless steel pan and hermetically sealed using an encapsulation press (TA Instruments, New Castle, DE). Samples were run against a reference pan containing air. Analysis was conducted over a temperature range of 25 - 110°C with a scanning rate of 5°C per minute.
**Fourier Transform Infrared (FTIR) Spectroscopy**

Samples of thin albumen from untreated, heat-pasteurized, and combination-pasteurized eggs were analyzed using a Digilab Excalibur 3500 FT-IR spectrometer (Digilab, Randolph, MA). Spectra were collected from 4000 - 700 cm\(^{-1}\) using an amorphous material transmitting infrared radiation (AMTIR) crystal, and 64 scans were combined. Background was collected before scanning samples (~20 mg) and crystal was cleaned with 75% ethanol between samples. Ten eggs from each treatment were analyzed in duplicate.

**Statistical Analysis**

Results of sulfhydryl group and lysozyme analyses were evaluated using SAS v. 9.1.3 software (SAS Institute, Inc., Cary, NC). Results were compared by general linear model with treatment as independent variable and effect as dependant variable; \( p < 0.05 \) was considered significant. Results of FTIR analysis were compared using Pirouette 3.11 software (Infometrix Inc., Woddinville, WA). Data was transformed to second derivative and a five-point polynomial-fit Savitzky-Golay function was used for the construction of soft independent modeling of class analogy (SIMCA) plot with interclass distance \( \geq 3.0 \) considered a significant difference. Discriminating power was used to identify peaks contributing to differences among treatments.
RESULTS

Sulfhydryl Groups

The conditions used in both heat only and combination pasteurization treatments were not sufficient to produce significant changes in the number of free sulfhydryl groups or disulfide bonds of treated albumen (Figure 5.3).

![Sulfhydryl groups in albumen of untreated eggs and eggs pasteurized by heat or heat/ozone combination.](image)

*Figure 5.3 Sulfhydryl groups in albumen of untreated eggs and eggs pasteurized by heat or heat/ozone combination.*

Error bars indicate standard error (n=15). No significant differences (p < 0.05) detected. Each bar represents total SH groups in reduced protein samples, with lower portion accounting for level in unreduced samples.

Lysozyme activity

Results of the lysozyme activity assay showed a slight, but not significant trend of increasing activity from untreated samples to combination pasteurized samples, with the highest activity in heat pasteurized samples (Table 5.1).
Table 5.1 Lysozyme activity of untreated and pasteurized eggs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lysozyme activity (U/ml albumen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>104 ± 7.0</td>
</tr>
<tr>
<td>Heat pasteurized</td>
<td>116 ± 7.0</td>
</tr>
<tr>
<td>Combination pasteurized</td>
<td>111 ± 7.0</td>
</tr>
</tbody>
</table>

**DSC**

Results of previous calorimetric studies on egg albumen agree that three bands are generally observed, representing ovotransferrin (~67.5°C), ovalbumin (~80°C) and S-ovalbumen (~85°C) (Rodriguez-Romo 2004, Donovan et al. 1975, Ferreira et al. 1997). These peaks were observed in thermograms obtained from all three treatments (Figure 5.4). Damage to ovotransferrin, one of the more heat sensitive albumen proteins, would be indicated by a shifting of the denaturation temperature of this band, an effect that was not observed. The conversion of ovalbumin to S-ovalbumin can be observed in the relative sizes of the peaks representing these proteins at 80 and 85°C, respectively. The thermograms obtained indicate that this transition, which is encouraged by heat exposure, was greater in combination pasteurized samples than in untreated eggs and greatest in heat pasteurized samples. Due to the overlapping nature of these two peaks, peak area quantitation is not possible.
Figure 5.4 Differential scanning calorimetry thermograms of albumin samples from untreated, heat pasteurized and combination pasteurized eggs.

FTIR

SIMCA class distance projections (Figure 5.5) display distinct clustering of treatments, indicating the existence of differences among them. These differences, however, according to interclass distance measurements (Table 5.2), are not significant. Upon comparing treatments, analysis of peaks providing discriminating power can elucidate the differences present. Differences between untreated and heat pasteurized eggs are mainly the result of a bands within the amide I region (Figure 5.6) which is indicative of conversion from α-helix to β-sheet structure induced by heating. Differences between untreated and combination pasteurized samples (Figure 5.7) are the result of peaks in both the amide I and amide II region, again most likely indicating a loss
of α-helical structure in treated samples due to heating. Differences between samples pasteurized with different methods (Figure 5.8) are attributed to vibrations of carbonyl (1740 cm⁻¹) and carboxyl (1400 cm⁻¹) groups. These peaks could indicate the production or exposure of acidic groups due to ozone treatment. This observation is supported by the previously demonstrated lower pH of albumen in eggs pasteurized by the combination process.
Figure 5.5 SIMCA class distance projections
Table 5.2 SIMCA interclass distances among treatments$^a$

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Heat pasteurized</th>
<th>Combination pasteurized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat Pasteurized</td>
<td>2.0888</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Combination pasteurized</td>
<td>1.4355</td>
<td>0.9478</td>
<td>N/A</td>
</tr>
</tbody>
</table>

$^a$Interclass distance $\geq 3.0$ is considered a significant difference

Figure 5.6 Discriminating power for differentiation of untreated and heat pasteurized albumen
Figure 5.7 Discriminating power for differentiation of untreated and combination pasteurized albumen

Figure 5.8 Discriminating power for differentiation of combination pasteurized and heat pasteurized albumen
DISCUSSION

Differences observed among treatments in this study were slight. While generally not significant, differences observed support a trend of greater albumen damage resulting from the heat alone process. Results did not indicate an increase in disulfide bonds of albumen treated with ozone. In a recent study (Fuhrmann et al. 2010), ozonation of shell eggs or albumen alone with levels of approximately 8% (wt/wt) for 60 minutes led to a significant decrease in free sulfhydryls, attributed to an increase in disulfide bonds due to the oxidative action of ozone, but that effect was not observed in the current work.

Lysozyme assay results confirm that the enzyme’s activity is not compromised by either the heat or combination pasteurization processes. The slight differences in lysozyme activity observed are in all likelihood the result of increased physical accessibility of the enzyme active site to the substrate used in the assay. Because of its high isoelectric point, lysozyme is the single known albumen protein with a positive charge within the environment of the egg. As a result, it is seldom found in isolation, but is most often complexed to one of the other major albumen proteins, often ovalbumen or ovomucin. We propose that the gentle heating treatments used in this work caused a partial unfolding of this complex, leading to exposure of active sites and consequent small increases in activity. It is expected that this effect would be observed up to the denaturation point of the lysozyme itself.

Among the heat labile albumen proteins, ovotransferrin seems to resist the pasteurization treatments. There was, however, an increase in the amount of S-ovalbumen relative to R-ovalbumen, with increasing severity of heat treatment. Another significant difference observed was in the secondary structure as displayed by FTIR
spectra. FTIR analysis can be utilized to give information regarding predominant secondary structures of proteinaceous samples. In protein samples consisting mainly of \(\alpha\)-helices, the amide I band on an FTIR spectrum should be observed in the vicinity of 1650 cm\(^{-1}\). A predominance of \(\beta\)-sheet structure causes this band to shift to approximately 1630 cm\(^{-1}\) (Jackson and Mantsch 1995). Bands in this range were principally responsible for differences detected between untreated and pasteurized samples using SIMCA projections. Previously, researchers have observed an increase in \(\beta\)-sheet content of albumen and other proteins after heating to temperatures up to 100°C using FTIR (Mine et al. 1990, Bonwell and Wetzel 2009), and analysis of FTIR spectra obtained in this study indicate that this conversion was increasingly affected by heat treatments of longer duration.

Based on these tests, it may be inferred that the probability of denaturation of individual heat labile proteins during egg pasteurization is low. On the contrary, previous observations of increased albumen opacity and whipping time in pasteurized samples seem to be the result of changes in protein composition and secondary structure. These changes (Chapter 4), were observed to a greater degree in heat pasteurized samples than combination pasteurized sample. This suggests that changes are the result of increased duration of heating. Results of this study verify the initial hypothesis of this work that a process using a shorter heating step would result in a less damaged final product. Differences in protein structure/enzyme activity due specifically to the application of ozone to shell eggs were not detected, further supporting the use of this sanitizer as a valid option for egg pasteurization.
REFERENCES


Chapter 6

Thermal Inactivation of *Salmonella enterica* Serovar Enteritidis in whole shell eggs, effect of inoculation site and refrigeration

ABSTRACT

Contamination of eggs with *Salmonella enterica* serovar Enteritidis is a major public health concern. Infection of laying hens causes bacterial cells to be deposited into egg contents during egg formation. Potential exists for cells to be found in either albumen or yolk and little research has been conducted regarding effect of cell location within shell eggs on resistance of these cells to processing. In the current research, *Salmonella* Enteritidis was inoculated into whole shell eggs and allowed to grow to high cell density. Inoculated eggs were heated for up to 40 minutes. The effect of inoculum location (albumen or yolk) on lethality of heat treatment was investigated. The effect of a period of refrigeration between incubation and treatment was also investigated. Cells present in egg yolk were inactivated more slowly than those in albumen due to the time needed for heat transfer to the interior regions of the egg. Refrigeration did not increase heat resistance of cells in albumen, but did lead to significantly less inactivation in cells grown in egg yolk. When temperature within the egg was monitored at the location of inoculum, comparison of inactivation at equivalent internal temperatures revealed similar levels of lethality, regardless of inoculation procedure.
INTRODUCTION

Experimental production of vertically contaminated chicken eggs has been demonstrated to be an unpredictable process. Several groups have attempted to produce vertically contaminated eggs by inoculation of laying hens (e.g., orally, intravenously or vaginally) with cultures of *Salmonella* spp. Their results reveal that the frequency of contamination is both low and intermittent (Humphrey et al. 1989, Okamura et al. 2001a, Okamura et al. 2001b, Gast and Holt 2000, Gast et al. 2004). Due to these difficulties, researchers have no options but to inoculate eggs directly with *Salmonella*. Simulation of naturally contaminated eggs through inoculation is an ongoing challenge for researchers.

While the majority of natural contamination is believed to occur in the albumen, (Humphrey et al. 1991) it has also been reported to occur, less frequently, in the yolk as well (Gast and Holt 2000, Murase et al. 2005, Murase et al. 2006). Some authors have reported migration of cells from albumen to yolk (Murase et al. 2006, Gast et al. 2006), but others were unable to reproduce this outcome (Fleischman et al. 2003). Due to this uncertainty, many of the studies seeking to quantify the heat resistance of salmonellae in eggs have involved inoculation and treatment of egg components separately, or of liquid whole egg removed from the shell (Garibaldi et al. 1969, Anellis et al. 1954, Humphrey et al. 1990, Michalski et al. 1999, Shah et al. 1991). Because of the unique shape and positioning of egg components, these results cannot easily be applied to whole eggs. Additionally, the majority of inactivation studies conducted in eggs or egg components involve inoculation immediately before treatment, a procedure that prevents cells from adapting to the environment of the egg and, depending on suspending medium, can encourage clumping of cells, leading to inaccurate results. The current work seeks to
determine whether the environment to which cells are adapted (albumen or yolk) plays a role in resistance to heating. The effect of refrigeration prior to processing will also be examined as a simulation of refrigerated storage during transport prior to processing.

MATERIALS AND METHODS

Eggs

Unfertilized, washed Grade AA large eggs were obtained from the farms of Hemmelgarn & Sons, Inc. (Coldwater, OH). Eggs were stored at 4°C until treatment and were treated within two weeks of laying. All eggs were weighed and only those weighing 2 oz ± 5% (56.7 ± 2.8 g) were used. Selected eggs were stored at room temperature for approximately 18 hours before treatment.

Culture Preparation

*Salmonella* Enteritidis ODA 99-30581-13, isolated from an egg source, was provided by the Ohio Department of Agriculture (Reynoldsburg, OH). The pathogen was cultured in tryptic soy broth (TSB; Criterion, Hardy Diagnostics, Santa Clara, CA) and incubated at 37°C for 24 hours. Aliquots of this culture were centrifuged at 4°C and 3020 x g for 10 minutes. Cell pellets were re-suspended in chilled phosphate buffer (0.2 mol l⁻¹, pH 7) and diluted as needed to achieve proper cell concentration for desired inoculum level.
Inoculation

Eggs were inoculated in the albumen, yolk, or on the vitelline membrane using slightly different procedures as follows. Shell eggs were punctured in the approximate center of the blunt side using a sterile 1.5 inch/20-gauge needle attached to a disposable 1 ml syringe (Becton Dickinson, Franklin Lakes, NJ). *Salmonella* Enteritidis cell suspension was introduced into eggs using a gas tight syringe coupled to 2 inch/22-gauge removable needle (Hamilton Company, Reno, NV) with rubber stopper affixed at set distance from needle point. For inoculation in albumen only, 100 μl of cell suspension (10^6-10^7 CFU ml^-1, verified by direct plating) was introduced into eggs at a depth of 4 mm, divided between two inoculation sites on opposite sides of the egg equator. For inoculation in yolk only, 10 μl of cell suspension (10^2-10^3 CFU ml^-1, verified by direct plating) was introduced into eggs at a depth of 17 mm. A third inoculation procedure (which will be referred to as the “diffusion” procedure) involved inoculation with 10 μl of cell suspension at a depth of 10 mm; subsequent incubation allowed natural migration of cells from albumen into yolk, with nearly equal population in each portion at the time of treatment (verified by plating). Inoculation site was wiped with ethanol (70% vol/vol) and allowed to dry for one minute. Holes were then sealed using Teflon tape (Cole-Parmer, Vernon, IL). Inoculated eggs were incubated at 30°C for 24 hours prior to treatment with optional storage step at 4°C for 24 hours. All eggs were equilibrated in a water bath set to 25°C for one hour prior to treatment.
Heat Treatment

Inoculated eggs were placed onto a custom-made stainless steel rack and heat treated by immersion in a circulating water bath (Thermo Corporation, Waltham, MA) set to 57°C. Internal temperature was monitored using an un-inoculated egg with thermocouple (Fluke, Everett, WA) wire inserted to depth of placed inoculum. Eggs were removed for analysis at five minute intervals for a total of 40 minutes. When removed from the water bath, inoculated eggs were immediately submerged in ice water until analysis. All analysis was completed in < 15 minutes after cessation of heating.

Enumeration of Surviving Cells

Eggs were broken aseptically and pooled in groups of three. Eggs with Salmonella present in both albumen and yolk were separated for enumeration using sterile, commercial egg separators. Egg contents were diluted 1:1 in 0.1% peptone water. Aliquots of this solution were diluted further as needed and plated on tryptic soy agar (TSA). Plates were incubated at 37°C for 24 hours.

Statistical Analysis

Data analysis was performed using SAS v. 9.1.3 software (SAS Institute, Inc., Cary, NC). Salmonella inactivation results were compared using an unpaired t-test with probability (p) value <0.05 considered significant. Internal temperatures required to illicit the initial one log CFU g⁻¹ reduction of Salmonella were compared using a mixed model with inoculation site, inoculation method and refrigeration as main effects.
RESULTS

Immediately before treatment, all inoculated eggs contained approximately $10^7$ CFU g$^{-1}$ of egg contents, regardless of inoculation method. Internal temperature in albumen and yolk throughout heating is displayed in Figure 6.1.

![Figure 6.1 Internal temperature in egg albumen and yolk during heating at 57°C. Error bars indicate standard deviation (n=3).](image)

After 40 minutes of heating at 57°C, all treatments displayed similar reduction (approximately 6.5 log) in *Salmonella* Enteritidis, with the exception of eggs which had been inoculated in the yolk and subjected to refrigeration (approximately 5.5 log reduction). Cells from this treatment were significantly more resistant ($p < 0.05$) to heating than those from all other treatments. Death curves for *Salmonella* Enteritidis inoculated in yolk are displayed in Figure 6.2. Refrigeration did not noticeably alter the inactivation of cells present in albumen only (data not shown).
Figure 6.2 Thermal inactivation of Salmonella Enteritidis inoculated in egg yolk and shell eggs heated at 57°C (treatment temperature). Error bars indicate standard error (n=9)

For much of the heat treatment, cells in the albumen were exposed to higher temperatures than those present in yolk (Figure 6.1). In order to accurately compare the thermal resistance of the cells in each portion of the egg, population of Salmonella Enteritidis must be compared according to internal temperature at the sight of inoculum placement. A comparison of the temperatures at which the first one log reduction was achieved in albumen and yolk is shown in Table 6.1.
Table 6.1 Internal temperature at inoculation site after 1-log reduction of *Salmonella* Enteritidis in inoculated shell eggs.

<table>
<thead>
<tr>
<th>Cell location</th>
<th>Inoculation method</th>
<th>Refrigeration</th>
<th>Temperature (°C) after 1-log reductiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yolk</td>
<td>Direct</td>
<td>No</td>
<td>51.9 ± 1.91A</td>
</tr>
<tr>
<td>Yolk</td>
<td>Direct</td>
<td>Yes</td>
<td>51.7 ± 3.27A</td>
</tr>
<tr>
<td>Albumen</td>
<td>Direct</td>
<td>No</td>
<td>50.6 ± 0.81A</td>
</tr>
<tr>
<td>Albumen</td>
<td>Direct</td>
<td>Yes</td>
<td>51.6 ± 2.06A</td>
</tr>
<tr>
<td>Yolk</td>
<td>Diffusion</td>
<td>No</td>
<td>48.2 ± 2.55A</td>
</tr>
<tr>
<td>Yolk</td>
<td>Diffusion</td>
<td>Yes</td>
<td>41.4 ± 9.45B</td>
</tr>
<tr>
<td>Albumen</td>
<td>Diffusion</td>
<td>No</td>
<td>50.2 ± 0.40A</td>
</tr>
<tr>
<td>Albumen</td>
<td>Diffusion</td>
<td>Yes</td>
<td>52.8 ± 0.40A</td>
</tr>
</tbody>
</table>

aDifferent superscripts within column indicate significant ($p < 0.05$) differences among values

When cells were allowed to diffuse from the albumen into the yolk, the resulting inactivation curve more closely resembled inactivation in the yolk than in the albumen, (Figure 6.3) despite the fact that cells were nearly equally split between the two egg portions. Refrigeration did not increase the resistance of cells present in both portions, but cells that had diffused into the yolk from the albumen displayed a one-log reduction in population at a temperature significantly lower than that required to elicit a one-log reduction in all other treatments (Table 6.1), indicating that these cells are less resistant to heat than cells initially placed into the yolk.
DISCUSSION

Previous work conducted on *Salmonella* in eggs and egg contents has focused on assigning a D-value to the bacterium. It has long been acknowledged that inactivation of microorganisms within complex food systems rarely follows a first-order kinetic. This observation makes D-value, a calculation based on linear inactivation, meaningless when measured in food systems. Values reported in the literature vary widely, with $D_{55}$-values for *Salmonella Enteritidis* in liquid whole egg ranging from under four to more than twelve minutes (Garibaldi et al. 1969, Humphrey et al. 1990, Michalski et al. 1999). Regardless of the inoculation procedure used, all inactivation curves obtained during the course of this research displayed shoulders and tailing, reinforcing the futility of linear modeling.
The results of this work indicate that habituation in the hostile environment of egg albumen does not confer additional heat resistance to *Salmonella* Enteritidis cells when compared to cells grown in the yolk. As expected, placement of cells in the yolk, because of that portion of the egg’s distance from the shell and correspondingly from the source of heat applied, increases the time needed to inactivate equivalent populations when compared to cells placed in the albumen, which reach lethal temperatures more quickly (Figure 6.2).

Incubation of inoculated eggs at 30°C for 24 hours was shown to facilitate the migration of bacterial cells from the albumen into the yolk, as demonstrated by the nearly equal counts in those portions of the egg following the “diffusion” inoculation procedure. While the addition of a refrigeration step to the incubation procedure did not increase the thermal resistance of cells in the albumen, or cells present in albumen and yolk at the same time, resistance was significantly increased in cells grown in the yolk only (Figure 6.1). On the contrary, when cells were allowed to migrate into the yolk from the albumen, added refrigeration led to the initiation of cell death at a lower temperature (Figure 6.1).

The findings of this study reinforce the importance of proper temperature control of newly laid eggs. It has been demonstrated previously that prompt refrigeration of eggs with salmonellae present in the yolk can delay or prevent cells from reproducing to high levels (Fleischman et al. 2003). It can also prevent the migration of cells present in albumen into the yolk (Gast et al. 2006, Fleischman et al. 2003). As the results of this study show, delay in refrigeration after introduction of cells into egg contents can
facilitate significant increases in bacterial population as well as, in some scenarios, contributing to the processing resistance of contaminants.

REFERENCES


APPENDIX A

Process development

INTRODUCTION

Previous work conducted in this laboratory by Dr. Luis Rodriguez-Romo had demonstrated the promise of a heat/ozone combination process to reduce internal Salmonella Enteritidis contamination in shell eggs using a benchtop scale setup. Comprehensive research had been conducted by Dr. Rodriguez-Romo to identify the parameters of the overall treatment that contributed significantly to the reductions observed (Rodriguez-Romo 2004). However, at the beginning of the project in its current iteration, the first task to be accomplished was the design of a similar process in a larger, pilot-scale setup. In order to accomplish this goal, a number of different parameters were varied in the early period of research to ascertain their effect on the overall efficacy of the final treatment process. The data obtained in that process development period is presented here.

Internal egg temperature

A range of temperatures have been used for heat treatment of eggs. There seems to be agreement among researchers that critical points for egg heating are times longer than 30 minutes and temperatures of 58°C or higher. When these parameters are met or exceeded, significant negative effects on the resulting egg can be observed. Previous treatments had employed a heat step (57°C for 25 minutes total heating time) believed to
result in a final internal temperature of approximately 56°C. In order to verify the internal temperature of eggs during heating, trials were conducted at 57, 58 and 59°C using uninoculated eggs with a thermocouple placed to the approximate depth of the vitelline membrane. In order to ensure that eggs reached an internal temperature of 56°C, but did not greatly exceed it, a heat treatment of 21 minutes (total heating time) at 57°C (water temperature) was initially selected.

**Ozone concentration**

The concentration of ozone used in this project is extremely high compared to the levels used for the treatment of most food products (Table 1.7). To verify the necessity of such a high concentration, experiments were carried out using a standardized heat treatment step and varied ozone concentrations. Egg preparation and heating was carried out as described in Chapter 2. Ozone treatment parameters tested are displayed in Table A.1. Pressure and ozone concentration during treatment at all levels of ozone is displayed in Figure A.1. Treated eggs were analyzed for surviving salmonellae by a modified most probable number method (described in Chapter 2). *Salmonella* inactivation data is displayed in Figure A.2.
### Table A.1 Ozone treatments used for assessment of concentration effect

<table>
<thead>
<tr>
<th>Vacuum (kPa)</th>
<th>Maximum ozone concentration (g m⁻³)</th>
<th>Pressure (kPa)</th>
<th>Holding time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>67.5</td>
<td>150 (~10%)</td>
<td>180</td>
<td>40</td>
</tr>
<tr>
<td>67.5</td>
<td>75 (~5%)</td>
<td>130</td>
<td>40</td>
</tr>
<tr>
<td>67.5</td>
<td>30 (~2%)</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>67.5</td>
<td>15 (~1%)</td>
<td>65</td>
<td>40</td>
</tr>
</tbody>
</table>

![Figure A.1](image)

Figure A.1 Pressure and ozone levels inside treatment chamber during treatment of shell eggs
Figure A.2 Log Reduction of *Salmonella* in whole eggs treated with heat + ozone combinations
Data analysis was performed using SAS v. 9.1.3 software. Results for all treatments (n = 24) were compared using an unpaired t-test with probability value <0.05 considered significant.
Error bars represent standard error.

Lower levels of ozone (1 and 2%), combined with heat, showed approximately 3-log reduction of *Salmonella* Enteritidis. This is approximately equivalent to the reduction afforded by the heat treatment utilized in this process (Chapter 2), suggesting that ozone at this lower level is either unable to penetrate the egg to the depth of the inoculum, or is of insufficient concentration to induce widespread lethality. Enhanced kill was observed when 5% ozone was employed, and reduction obtained using this treatment was not statistically different from that obtained from use of 10% ozone. There does, however, appear to be a trend of increasing inactivation with increasing concentration. From this data, 10% ozone was selected as the standard for treatments going forward.
Gas agitation

In initial experiments conducted by Dr. Rodriguez-Romo, ozone treatment was conducted in a small vessel placed on an orbital shaker. The agitation of the vessel during treatment was thought to ensure homogeneous distribution of ozone gas within the treatment chamber. In the design of the pilot-scale setup, (Figure 2.2) a fan was included at the rear of the vessel to accomplish this function. The necessity of gas agitation for ozone lethality on internal Salmonella contamination was investigated in a series of experiments in which all methods were identical to those described in Chapter 2, with the exception of the absence of gas agitation. This treatment resulted in an average reduction of 3.9 ± 1.1 log Salmonella Enteritidis per egg, which was not significantly different from the 4.2-log reduction achieved with gas agitation. Because use of the fan complicated the mechanics of the vessel operation, contributing to ozone leaks, further experiments were conducted without agitation of gas.

REFERENCE

APPENDIX B

*Salmonella enterica* serovar and strain comparison

**ABSTRACT**

Concern regarding the health threat posed by antibiotic resistant bacteria has risen greatly in recent years. This risk is intimately tied to the food industry due to the widespread use of antibiotics on food animals and the transmission of pathogens from food to humans. Because genes conferring antibiotic resistance are often encoded on mobile DNA elements, the possibility exists for acquisition of resistance in nearly any species, including processing-resistant pathogenic strains. Eighteen *Salmonella enterica* serovars, including ten *Salmonella Enteritidis* egg isolates, were tested for tolerance to heat (57°C for up to 60 minutes), and ultra high pressure (400 and 500 MPa for one minute). Isolates were also tested for susceptibility to four antibiotics (AMP, CHL, KAN and TET) via the dilution plating method.

No significant differences in thermal tolerance ($p < 0.05$) were observed among isolates. At 400 MPa, *Salmonella Anatum* and *Salmonella Muechen* were significantly less resistant to pressure than *Salmonella Kentucky* and *Salmonella Enteritidis* ODA 99-30581-13. At 500 MPa, *Salmonella Muechen* was significantly more sensitive than *Salmonella Enteritidis* ODA 99-30581-36 and *Salmonella Kentucky*. Serovars were classified as susceptible, intermediate or resistant to antibiotics according to criteria published by the Clinical and Laboratory Standards Institute (2005). Three strains of
**INTRODUCTION**

Over the course of the past two decades, isolation of bacteria possessing resistance to antibiotics has increased dramatically. This trend encompasses not only clinical and environmental samples, but also applies to isolates obtained from foods. Foods of animal origin are common sources of these isolates due to the fact that antibiotics are used not only to treat and prevent animal illness, but are also administered constantly in feed at low levels in order to promote growth (Teuber 1999). Efficiency of horizontal gene transfer within the *Enterobacteriaceae* family has been well documented (Martinez-Freijo et al. 1998). It has also been established that common methods of food processing and preservation can increase the rate of transmission of plasmids carrying genes for antibiotic resistance (McMahon et al. 2007).

Within the *Salmonella* genus, intrinsic differences in susceptibility to common food processing methods has been confirmed (Humphrey et al. 1995, Metrick et al.
Salmonella Senftenberg 775W is particularly well known for its uncharacteristically high thermal tolerance. The acquisition of antimicrobial resistance by a serovar with naturally higher resistance to processing methods would present the threat of increased processing survival coupled with establishment of a less treatable infection in susceptible population and possible subsequent transfer of resistance to commensal flora. The goal of this research is to determine whether a connection between processing and antibiotic resistance can be made among several salmonellae.

MATERIALS AND METHODS

Culture Preparation

Salmonella enterica cultures (various serovars and strains, Table B.1) were cultured in tryptic soy broth (TSB; Criterion, Hardy Diagnostics, Santa Clara, CA) incubated at 37°C for 24 hours.
Table B.1 *Salmonella enterica* serovars and strains analyzed

<table>
<thead>
<tr>
<th>Serovar and strain</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> Enteritidis ODA 99-30581-6</td>
<td>Enteritidis 6</td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis ODA 99-30581-13</td>
<td>Enteritidis 13</td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis ODA 99-30518-19</td>
<td>Enteritidis 19</td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis ODA 99-30581-20</td>
<td>Enteritidis 20</td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis ODA 99-30581-30</td>
<td>Enteritidis 30</td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis ODA 99-30581-36</td>
<td>Enteritidis 36</td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis ODA 99-30581-38</td>
<td>Enteritidis 38</td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis ODA 99-30581-43</td>
<td>Enteritidis 43</td>
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<tr>
<td><em>Salmonella</em> Enteritidis ODA 99-30581-45</td>
<td>Enteritidis 45</td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis ODA 99-30581-48</td>
<td>Enteritidis 48</td>
</tr>
<tr>
<td><em>Salmonella</em> Muenchen ATCC 8388</td>
<td>Muenchen</td>
</tr>
<tr>
<td><em>Salmonella</em> Anatum*</td>
<td>Anatum</td>
</tr>
<tr>
<td><em>Salmonella</em> Kentucky*</td>
<td>Kentucky</td>
</tr>
<tr>
<td><em>Salmonella</em> Heidelberg*</td>
<td>Heidelberg</td>
</tr>
<tr>
<td><em>Salmonella</em> Senftenberg OSU 836</td>
<td>Senftenberg</td>
</tr>
<tr>
<td><em>Salmonella</em> Typhimurium LT2</td>
<td>T. LT2</td>
</tr>
<tr>
<td><em>Salmonella</em> Typhimurium OSU 228</td>
<td>T. OSU 228</td>
</tr>
<tr>
<td><em>Salmonella</em> Typhimurium DT109</td>
<td>T. DT109</td>
</tr>
</tbody>
</table>

ODA denotes Ohio Department of Agriculture  
*Additional information unavailable

**Heat Treatment**

For heat treatment experiments, cultures grown in TSB were subsequently transferred to MacConkey broth (Alpha Biosciences, Baltimore, MD) and incubated for additional 24 hours in an orbital shaker (Lab-Line, Mumbai, India) set at 37°C and 175 rpm. Aliquots of this culture were centrifuged at 4°C and 3020 x g for 10 minutes and re-suspended in chilled phosphate buffer (0.2M, pH 7). Concentrated cell suspension (100 μl, ~40x, 10^{11} CFU/ml) was deposited into thin-walled PCR tubes and heated in a PCR thermocycler set to 57°C for 0, 30, or 60 minutes. Following heat treatment, suspension was spread plated onto tryptcase soy agar (TSA) and incubated at 37°C for 24 hours. Four *Salmonella* strains (*Salmonella* Muenchen, *Salmonella* Enteritidis 2, *Salmonella* Typhimurium DT109, and *Salmonella* Heidelberg) were studied in more detail. Cell
suspensions were prepared and heated as described, but samples were taken at 0, 2, 5, 10, 20 and 30 minutes to gain better insight into inactivation kinetics.

**Pressure Treatment**

Aliquots of TSB culture were centrifuged at 4°C and 3020 x g for 10 minutes and re-suspended in chilled phosphate buffer (0.2M, pH 7). All necessary dilutions were made in sterile 0.1% peptone water. One ml aliquots were transferred to polyethylene bags which were heat sealed and vacuum packaged before treatment. Packaged samples were subjected to cold shock by storage on ice for 30 minutes. Samples were submerged in ethylene glycol and treated at 400 MPa or 500 MPa for one minute. Treated samples were spread plated on TSA and incubated at 37°C for 24 hours.

**Antibiotic Susceptibility Testing**

All strains were tested for susceptibility to ampicillin, tetracycline, chloramphenicol and kanamycin. The dilution plating method, previously described by the Clinical and Laboratory Standards Institute (CLSI) was used. Minimum inhibitory concentrations were compared to published CLSI standards for determination of resistance.

**Statistical Analysis**

Data analysis was performed using SAS v. 9.1.3 software. Results for heat and pressure treatments were compared using analysis of variance with probability value < 0.05 considered significant.
RESULTS

Heat Treatment

Variations in thermal resistance of *Salmonella* serovars are shown in Figure B.1. One serovar, *Salmonella* Muenchen, appeared to be the least resistant to heat treatment, as it was completely inactivated by 30 minutes of heating. Inactivation kinetics varied greatly among strains tested (Figure B.2). A comparison of population by sampling time is displayed in Table B.2.

![Figure B.1 Log reduction of *Salmonella* after heat treatment at 57C for 30 or 60 minutes](image-url)
Figure B.2 Inactivation of Salmonella enterica serovars heated at 57°C
n=3, error bars denote standard error

Table B.2 Differences in population of Salmonella serovars (log CFU ml⁻¹) during 30 minute heating at 57°C

<table>
<thead>
<tr>
<th>Serovar</th>
<th>0 min</th>
<th>2 min</th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muenchen</td>
<td>10.6Ａ</td>
<td>5.05ＡＢ</td>
<td>2.12Ａ</td>
<td>1.43Ａ</td>
<td>1.43Ａ</td>
<td>1.33Ａ</td>
</tr>
<tr>
<td>Heidelberg</td>
<td>9.78Ａ</td>
<td>3.62Ａ</td>
<td>1.67Ａ</td>
<td>1.33Ａ</td>
<td>1.33Ａ</td>
<td>1.33Ａ</td>
</tr>
<tr>
<td>Typhimurium DT109</td>
<td>10.6Ａ</td>
<td>5.87Ｃ</td>
<td>6.09Ｂ</td>
<td>5.68Ｂ</td>
<td>4.37Ｂ</td>
<td>2.99Ｂ</td>
</tr>
<tr>
<td>Enteritidis</td>
<td>10.7Ａ</td>
<td>6.27ＢＣ</td>
<td>5.73Ｂ</td>
<td>3.73Ｂ</td>
<td>3.05ＡＢ</td>
<td>1.65ＡＢ</td>
</tr>
</tbody>
</table>

Values within columns with different superscripts are significantly different (p < 0.05)

Pressure Treatment

Differences in resistance to high pressure among serovars were observed (Figure B.3). At 400 MPa, log reductions ranged from ~1 – 3 log ml⁻¹ for all serovars and strains tested. Salmonella Kentucky and Salmonella Enteritidis 13 were significantly more resistant to treatment than Salmonella Muenchen and Salmonella Anatum. At 500 MPa, reductions were greater, ranging from just under 3 log to almost 5 log ml⁻¹. At the higher
pressure, *Salmonella* Enteritidis 36 and *Salmonella* Kentucky were significantly more resistant than *Salmonella* Muenchen.

Figure B.3 Log reduction of *Salmonella* after treatment with UHP
(A) 400 MPa, 1 min, 25°C. (B) 500 MPa, 1 min, 25°C.
Error bars represent standard error.
* Significantly more resistant (p<0.05) than **
Antibiotic Susceptibility Testing

Results of antibiotic susceptibility testing, displayed in Table B.3, were varied. All *Salmonella* Enteritidis strains tested were susceptible to the four antibiotics used, as were *Salmonella* Muenchen and *Salmonella* Anatum. *Salmonella* Kentucky was the only serovar to display a unique resistance profile. The most resistant serovars were *Salmonella* Heidelberg, *Salmonella* Senftenberg, *Salmonella* Typhimurium (LT2, OSU228 and DT109), all of which shared the same profile, displaying susceptibility only to kanamycin.

Table B.3 Susceptibility of *Salmonella* to antibiotics
AMP = Ampicillin, CHL = Chloramphenicol, KAN = Kanamycin, TET = Tetracycline
S = Susceptible, I = Intermediate, R = Resistant (Clinical and Laboratory Standards Institute 2008)

<table>
<thead>
<tr>
<th>Strain</th>
<th>AMP</th>
<th>CHL</th>
<th>KAN</th>
<th>TET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enteritidis (all strains)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Muenchen</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Anatum</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Kentucky</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>S</td>
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<td>Heidelberg</td>
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<td>Senftenberg</td>
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<td>R</td>
</tr>
<tr>
<td>T. LT2</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>T. OSU228</td>
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<td>R</td>
</tr>
<tr>
<td>T. DT109</td>
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</tr>
</tbody>
</table>
DISCUSSION

No definitive relationship among intrinsic resistances to processing techniques and antimicrobial agents was observed. However, existence of significant strain variability was confirmed with regard to UHP treatment (Figure B.2) and profiles of antibiotic resistance (Table B.2). Salmonella Muenchen ATCC 8388 was highly sensitive to pressure treatment at both 400 and 500 MPa and was also susceptible to all four antibiotics tested. Investigation into heat inactivation kinetics also demonstrated that Salmonella Muenchen was significantly more heat sensitive than Salmonella Typhimurium DT109 (Figure B.2 and Table B.2). Salmonella Kentucky was resistant to pressure treatment at both 400 and 500 MPa and also displayed the greatest resistance to kanamycin among all strains. These results suggest that further study regarding a relationship between antibiotic resistance and tolerance of UHP treatment may be warranted. All strains of Salmonella Enteritidis examined (egg isolates) were sensitive to antibiotics tested, suggesting the possibility of lack of expression of resistance due to the absence of selective pressure in the laboratory setting. All strains of Salmonella Typhimurium as well as Salmonella Heidelberg and Salmonella Senftenberg OSU 836 displayed resistance to multiple antibiotics. These patterns are consistent with a common phenotype which is comprised by resistance to sulphonamides and streptomycin in addition to ampicillin, chloramphenicol and tetracycline (Humphrey et al. 1995).
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