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ANTIMICROBIAL EFFECT OF HEAT AND OZONE ON *SALMONELLA ENTERITIDIS* IN TABLE EGGS

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SUMMARY

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. One hundred chicken table eggs were used for experimental treatments, fifty whole shell eggs were inoculated with small populations of *Salmonella enteritidis* (10 µl, approx. 10^7 CFU /ml) near the egg vitelline membrane.Ten eggs used as control positive, twenty eggs were subjected to heating (57°C for 21 and 40 min), ten eggs were subjected for ozone treatment (at concentration of 70 µg/ml for 40 min), ten eggs subjected to a combination of both treatments heat(57°C for 40 min) and ozone at concentration of 70 µg/ml for 40 min. Heat, Ozone and combination treatments inactivated (1.1, 3·1), 0·11, and 4·2 log *Salmonella enteritidis* per egg, respectively and thirty shell eggs without inoculation were used for studying effect of different treatment on sensory/visual evaluation. Twenty shell eggs externally contaminated with *Salmonella enteritidis* ($8x10^5$ CFU/ eggshell) divided into two groups, first group used as control positive, second group was treated with gaseous ozone (70µg /ml for 40 min). Ozone treatment reduced *Salmonella* on the surface of shell eggs by5.9 log/egg. Survivors were detected only in 10% from samples after an enrichment process.

INTRODACTION

Chicken eggs are a unique well-balanced source of nutrients in the human diet. Egg proteins have a high biological value, and are often used as the standard to compare the quality of other proteins in foods. In addition, eggs contain unsaturated fatty acids, iron, phosphorus, trace minerals, and vitamins **(Stadelman, 1995a; Watkins, 1995)**. Shell eggs consist of 9.5% shell, 63% albumen, and 27.5% yolk **(Li-Chan *et al.*, 1995)**.The egg shell is composed of 94% calcium carbonate, 1% magnesium carbonate,1%calcium phosphate, and 4% protein The shell

is a porous structure (~ 10,000 pores/shell), has an average thickness of 0.31 mm, and is covered by the cuticle, which is a protein-rich coating that constitutes the most external layer of the egg **(Stadelman, 1995b; Okubo *et al.*, 1997**).

Salmonella is one of the most prevalent pathogens associated with food-borne illness **(Lynch *et al.* 2006)**. In recent decades, proportion of salmonellosis attributed to *Salmonella enterica* subspecies *enterica* serovar *enteritidis* (*Salmonella enteritidis)*, relative to other *Salmonella* serovars, has increased. The most common food source of *Salmonella Enteritidis* is eggs. Shell eggs are commonly contaminated in a vertical manner during egg formation in colonized hen oviduct, or horizontally via migration of salmonellae through the egg shell after contact with contaminated substances such as hen faeces **(Guard-Petter 2001; Grijspeerdt *et al.* 2005; Lynch *et al.* 2006).** Internally contaminated eggs, obtained from naturally infected hens, may contain ≤10 *Salmonella enteritidis* per egg and the pathogen is most likely localized outside the vitelline membrane or in the albumen surrounding it **(Humphrey 1994; Humphrey *et al.* 1991; Poppe *et al.* 1992**). 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).** Although shell eggs are sanitized by washing and exposed to rapid chilling, these methods do not destroy Salmonella if it is harbored inside shell eggs (**Catalano and Knabel, 1994).**

Public health concerns regarding contamination of fresh eggs with *Salmonella enteritidis* prompted USDA approval of a thermal process to eliminate this microorganism in shell eggs **(USDA, 1997**). Pasteurization of eggs (in shell pasteurization) is a commercially available process, which consists of extended heating of shell eggs by immersion in water baths at 55-60ºC or by hot air in convection ovens **(Schuman *et al.*, 1997; Zeidler, 2001a; Brackett *et al.*, 2001).** 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 (US-FDA) 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 and Yousef 2004; Rodriguez-Romo *et al.* 2007).**

There for our research aimed to investigate the possibility of reducing or eradicating *Salmonella enteritidis* in experimentally inoculated whole shell eggs that are sequentially treated with heat and gaseous ozone and assessing the effectiveness of combination processes, to provide raw shell eggs free from *Salmonella enteritidis* have the same sensory and functional properties as untreated eggs and will be suitable for consumption as a ready –to-eat food.

MATERIALS AND METHODS

Egg preparation

One hundred chicken table eggs were obtained from a farm one day old (45–46 g/egg) were used for experimental treatments and Eggs were stored at 4°C. Selected eggs were stored at room temperature for approx. 2 h before being scrubbed individually with a plastic brush under cool running tap water. Washed eggs were soaked in ethanol (70% v/v) for 30 min to sanitize shells. Eggs were then placed in previously sterilized egg trays and allowed to dry at room temperature for 40 min, approximately, prior to inoculation. Eggs divided into 4 groups, each group contain ten eggs, first groups control positive without treatment, second groups treated with heat, third groups, treated with ozon and forth group treated with heat-ozone combination

Culture preparation

*Salmonella enteritidi*s, was obtained from Food Hygiene Dep. In Animal Health Research Institute (AHRI).The pathogen was cultured in tryptic soy broth (TSB) and incubated at 37°C for 24 h. 0.15ml of overnight culture was transferred to 150 ml MacConkey broth and

incubated at 37°C for 24 h, cell culture were centrifuged at 8000 rpm for 10 min. Supernatent was discarded and cells were re-suspended in sterile phosphate buffer salin and combined for a final concentration of approx. 10^{11} CFU ml^{-1}; this cell suspension was diluted to achieve final cell concentration about $3x10^7$ **(perry et al, (2008).**

Inoculation

Dried, sanitized shell eggs were divided into two groups, first group fifty shell eggs were inoculated with *Salmonella* according to **(Rodriguez-Romo 2004).** Clean and sanitized eggs were punctured in the approximate centre of the blunt side using a sterile needle. *Salmonella enteritidis* cell suspension (10 µl, approx. 10^7 CFU ml^{-1}, verified by direct plating) was introduced into eggs near the vitelline membrane using a sterile needle. Inoculation site was wiped with ethanol (70% v/v) and allowed to dry for one minute. Holes were then sealed using fast drying glue. Internal inoculation level of S*almonella enteritidis* was 5×10^5 CFU per egg.

Second group represented by twenty eggs were dipped for approximately 10 sec into stirred *Salmonella enteritidis* cell suspension prepared as described previously. Contaminated shell eggs were transferred to sterile carton trays and permitted to dry for approximately 30 min before treatments **(Rodriguez-Romo 2004).** Shell eggs divided into two groups, first group used as control positive, second group was treated with gaseous ozone. *Salmonella enteritidis* count on externally contaminated shell eggs was 8.0 □ 10^5 CFU/g.

Heat treatment

Inoculated eggs were placed on a hot air oven (Venticell MM Medcenter Einrichtungen, German) set to 57°C supplied with calibrated thermometer for 21 and 40 min. Immediately after treatment, eggs were either stored at 4°C (for heat alone samples) or transferred to ozonation vessel for further treatment. Each treatment applied on ten eggs.

Ozone treatment

Eggs were subjected to ozone treatment. Gaseous ozone was produced from pure oxygen by an ozone generator (Humazon, Humaras, German). The output of this generator is an ozone-oxygen mixture that will be referred to as 'ozone gas' or 'gaseous ozone' throughout this manuscript. Generator output was pumped to a custom treatment vessel to a maximum ozone concentration of 70 □g/ml. Eggs were subjected to static treatment for 40 min. Treated eggs were removed from the vessel and held at 4°C for 18 h to insure exhaustion of ozone residues.

Enumeration of surviving cells

Inoculated eggs and eggs treated with heat, ozon and ozone–heat combination were analyzed for surviving *Salmonella enteritidis.* Homogenization was done with buffered peptone water; serial dilutions were made using peptone water 0.1%, and were plated on Xylose Lysine Desoxycholate agar (XLD). Plates were incubated at 37 ^{o} C for 24 hr and enumeration were conducted. Remaining volume of original dilution was incubated at 37°C for 18h then 0.1 ml of pre-enrichment broth was transferred into a tube containing 10 ml Rappaports Vassiliadis and incubated at 41.5 ^{o}C for 24 hr then streaked onto (XLD) and incubated at 37 ^{o} C for 24 hr to confirm presence of *Salmonella*. (**ISO, 2002)**.Typical *Salmonella* colonies on XLD were subjected to biochemical and serological identification.

Interior egg quality

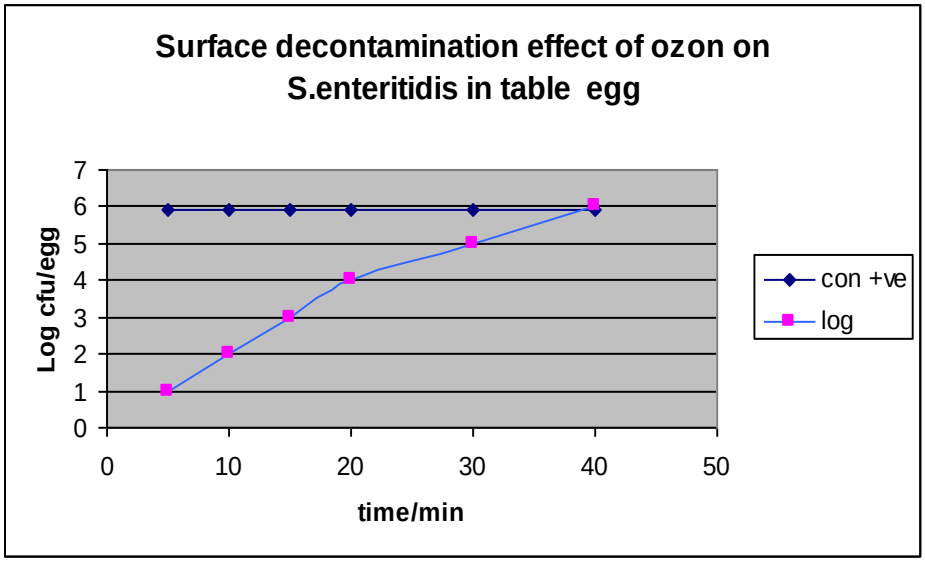
Thirty shell eggs were used for visual evaluation included the smell, test, color of each of white and yolk of eggs, and the white turbidity.

RESULTS AND DISCUSSION

Table (1)Inactivation of *S.enteritidis* in shell eggs after various treatment

Treatment	Treatment condition	Average decrease in log cfu/egg	Positive /Total
Inoculated egg	non treated	_	10/10
Heat	57°C for 21 min	1.1	10/10
	57°C for 40 min	3.1	10/10
Ozone (O_3)	70µg/ml for 40 min	0.11	10/10
Heat and ozone	57°C for 40 min 70µg/ml for 40 min	4.1	10/10

Fig (1)



Antimicrobial effect of heat on *S.enteritidies* in table eggs

Pasteurization of shell eggs is a technology that has been approved by the U.S.Department of Agriculture (USDA) for commercial application (USDA, 1997).The pasteurization process consists of prolonged heating of shell eggs by immersion treatments in water baths at 56-62□C or by hot air in convection ovens **(Schuman, 2000; Zeidler, 2001a; Brackett *et al.*, 2001).** Results represented in table (1) reported that heating internally contaminated shell eggs by 5.5 log *Salmonellae enteritidis* by using hot air oven at 57□C for 21 min resulted in microbial reductions by 1.1log/egg, while their treatment with 57□C for 40 min, resulted in microbial reductions 3.1 log/egg.

As most naturally contaminated *Salmonellae enteritidis* eggs contain count less than 10 cell and freshly laid eggs are very rarely reported to harbor more than a hundred of *Salmonellae enteritidis* **(Humphrey *et al*, 1989, 1991, Gast and Beard 1992, Gast and Holt, 2000).** In the light of these facts, egg pasteurization that successfully eliminated S.E and provide a wide margin of consumer safety.**Hou *et al.* (1996)** reported that *Salmonella enteritidis* was effectively inactivated by 5 log10, in the yolk of shell eggs, after treatment in a hot-air oven at 55□C for 3 h.

In addition, these researchers reported that heating internally contaminated shell eggs in water at 57□C for 25 min, followed by their treatment with hot air at 55□C for 60 min resulted in microbial reductions by 7-log10. While **Schuman *et al.* (1997**) found that heated shell eggs inoculated in the yolk with 107 *Salmonella enteritidis,* in water at 57 and 58 □C and reported microbial reductions by □.5.6 log10 after 55 min and □.5.8 log10 after 43 min, respectively, egg showed variable degrees of affection concerning physical properties as Haug units, yolk index and pH. Also **Brackett *et al*. (2001)** stead that heated yolk-contaminated shell eggs, by convection currents of humid air, and reported D-values of 5.4-6.1 min for a salmonellae cocktail treated at 57.2□C. Egg heating using this procedure for □.70 min resulted in non-detectable levels of the microorganism.

Rodriguez-Romo (2004) Found that contaminated eggs (~ 106 CFU/g egg), were heated by immersion in water at 57, 58, or 59ºC reduced *Salmonella* by 4.8, 5.1, and 5.4 log10, respectively, during 30-40 min heating. While **Perry *et al*. (2008)** Reported that whole shell eggs were inoculated with small populations of *Salmonella enteritidis* ($8{\cdot}5 \times 10^4$–$2{\cdot}4 \times 10^5$ CFU per egg) near the egg vitelline membrane. Eggs were subjected to immersion heating (57°C for 21 min), inactivated 3·1log *Salmonella enteritidis* per egg.

Antimicrobial effect of Ozon on *S. enteritidies* in table eggs

Recently, in the United States, the Food and Drug Administration approved the use of ozone in its gaseous and aqueous phase as an antimicrobial agent in food **(CFR, 2001).**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 and Yousef 2004; Rodriguez-Romo *et al.* 2007).**

Destruction of *Salmonella enteritidis* in shell egg by ozon gas treatment was demonstrated (table 1). Treatment shell eggs inoculated with *Salmonellae enteritidis* (5×10^5 CFU per egg) were treated by ozonation at concentration of 70□g/ml for 40 min). Treatment with ozone alone resulted in only 0.11 log reduction, a difference that is not significant from the untreated control egg the same resulted obtained by **Perry *et al.*** (**2008)** who reported that whole shell eggs were inoculated with *Salmonella enteritidis* ($8{\cdot}5 \times 10^4$–$2{\cdot}4 \times 10^5$ CFU per egg) were subjected to ozone treatment (140 g ozone m^{-3} and 184–198 kPa for 40 min) inactivated 0·11 log *Salmonella enteritidis* per egg.Decontamination of *Salmonella enteritidis* on the surface of table eggs was identified in (fig.1) Shell eggs externally contaminated with *Salmonella enteritidis* ($8x10^5$CFU/ eggshell) were treated with gaseous ozone (O3) (70□g /ml for 40 min) reduced *Salmonella* on the surface of

shell eggs by 5.9 log/egg. Survivors were detected only in 10%from samples (1/10) after an enrichment process.

The feasibility of gaseous ozone to reduce the number of microorganisms on the shell surface, of *Salmonella enteritidis* in particular, of avian hatching eggs was investigated. Shell eggs were externally contaminated with *Salmonellae enteritidis* to contain either 10^2–10^4 or 10^5–10^6 cfu/shell. Subsequently, the eggs were exposed to several ozone concentrations ranging from 0.5% to 5% wt/wt in combination with two relative humidities (< 30, > 70%) at room temperature. Exposure times varied between 20 minutes and 24 hours. A complete inactivation of 10^2–10^4cfu S.E./egg shell was reached by using an ozone concentration of 1% (wt/wt) for 120 min. Considering higher concentrations of S.E. on the shell ozone treatment caused approximately a 6 $\log_{10}$ reduction. This demonstrates that gaseous ozonation is suitable for applications in hatcheries provided that high-power ozone generators are available. The parameters should be verified in large ozone cabinets. (**Braun *et al*., 2011).**

Investigations into various alternative techniques for decontamination of the surfaces of artificially contaminated shell eggs were carried out. Ionized air, exposure to ozone in a dry atmosphere and use of a commercial herbal antibacterial product were not effective. Application of ozone in a humid environment was only partially effective but a commercial ionized water anolyte was highly effective in eliminating *Salmonella* from egg surfaces**. (Davies and Breslin 2003).**

Ozone has strong antimicrobial activity against bacteria, fungi, viruses, protozoa, and spores from bacteria and fungi **(Khadre *et al.*, 2001)**. The mechanisms involved in microbial inactivation by ozone are complex, and some reports indicate that ozone acts against unsaturated lipids in the microbial cell envelope, lipopolysaccharides in Gram-negative bacteria, intracellular enzymes, and genetic material (**Khadre *et al.*, 2001; Kim *et al.*, (2003**). It appears that ozone reacts with the double bonds of unsaturated lipids in the cell envelope, causing leakage of cell contents and eventually microbial lysis **(Scott and Lesher, 1963). Murray *et al.* (1965) i**ndicated that, ozone initially targets

lipoprotein and lipopolysaccharide layers of Gram-negative bacteria, changing membrane permeability and consequently leading to cell death.

Antimicrobial effect of heat and Ozon on *S.enteritidies* in table eggs
Cox *et al.* (1995) claimed that combined treatments heating shell eggs at 59.4ºC followed by application of ozone reduced the level of microorganisms in shell eggs and extended the shell-life of the product. Combined treatments using heat and ozone gas reported in table (1). Whole shell eggs were inoculated with *Salmonella enteritidis* (5×10^5 CFU per egg) were treated by heat at 57°C for 40 min followed by ozonation at concentration of 70□g/ml for 40 min). Combination treatments inactivate *Salmonella enteritidis* 4·2 log per egg.

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. **Rodriguez-Romo (2004**), the current study reports the synergy between ozone and heat when a smaller population of *Salmonella* is introduced in eggs. For example, shell eggs heated at 57□C for 25 min, subsequently placed under vacuum (-7 to -10 psig), and treated with O3 at 10 psig for 40 min, resulted in □6.3log10 *Salmonella* reduction within shell eggs. Egg quality was not drastically affected after treatments with heat and O3.**Perry *et al.*** (**2008)** reported that inoculated shell eggs with small populations of *Salmonella enteritidis* (5log per egg) were subjected to immersion heating (57°C for 21 min) and ozone treatment (vacuum at 67·5 kPa, followed by ozonation at a maximum concentration of approx. 140 g ozone m^{-3} and 184–198 kPa for 40 min. Combination treatments inactivated 4·2 log *Salmonella enteritidis* per egg. Survivors were detected after an enrichment process or enumerated using modified most probable number technique.

Interior egg quality
Eggs heated in hot air oven and eggs exposed to ozone gas have the same sensory evaluation as the standard egg. The yolk colour was

golden yellow while the albumen was clear. So egg quality was not affected after treatments with heat and O3. . Examination of physical properties of egg white after pasteurization indicated that the overall functionality of pasteurized shell eggs was acceptable under the heating condition **Hou *et al.* (1996).** While **Schuman *et al.* (1997**) found that heated shell eggs inoculated in the yolk with 107 *Salmonella enteritidis*, in water at 57 and 58 □C for 55 min and 43 min.Egg showed variable degrees of affection concerning physical properties as Haug units, yolk index and pH.

CONCLUSIONS

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 the heat or ozone treatment alone. Also zone can be used in surface decontamination of *Salmonella enteritidis* on the of table eggs.

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تاثير الحرارة والأوزون على تقليل ميكروب السالمونيلا انترتيدس فى بيض المائدة

سلوى رجب سليمان حجازى

قسم الرقابة الصحية على الاغذية- معهد بحوث صحة الحيوان -الدقى

الملخص

البيض هي الوسيلة الأكثر شيوعا لحالات العدوى البشرية بميكروب السالمونيلا انترتيدس وسجلت سنويا عديد من حالات التسمم بميكروب السالمونيلا انترتيدس ذات الصلة ببيض المائدة،على الرغم من الجهود المبذولة للحد من التلوث بميكروب السالمونيلا ، بما في ذلك استخدام البسترة الحرارية للبيض ومنتجاتة. كما وجد ان استخدام غاز الأوزون او الجمع بين استخدام البسترة وغاز الأوزون يؤدى الى إنتاج بيض أكثر أمانا من استخدام الحرارة وحدها.و قد اجرى البحث علي 100بيضة من بيض المائدة لدراسة تاثير استخدام البسترة الحرارية و استخدام غاز الأوزون او الجمع بين استخدام البسترة وغاز الأوزون على ميكروب السالمونيلا انترتيديس في بيض المائدة.

وقد تم تلقيح ٥٠ بيضة بميكروب السالمونيلا انترتيدس، حوالي10^7 خلية / مل بالقرب من الغشاء المحي للبيض.و تم استخدام ١٠بيضات كضابط ايجابى لميكروب السالمونيلا. وقد تم تعريض ٢٠ بيضة للبسترة عند درجة حرارة ٥٧ ْم لمدة ٢١ و ٤٠ دقيقة، و 20 بيضة تم تعريضها الى غاز الأوزون ٧٠ ميكرولتر / مل لمدة ٤٠ دقيقة ، ١٠بيضات تم علاجها باستخدام المزج بين الحرارة عند درجة حرارة٥٧ ْم لمدة ٤٠ دقيقة، وغازالأوزون ٧٠ميكرولتر / مل لمدة ٤٠ دقيقة. فوجد ان استخدام هذة المعلاجات تؤدى الى انخفاض في ميكروب السالمونيلا انترتيدس بمقدار(١.١، 1 .3)، ٠.١١ ، و2. 4 لو على التوالي.

تم استخدام ٣٠بيضة كضابط سلبي لميكروب السالمونيلا لدراسة تاثيرالمعالجات المختلفة علي الخواص الحسية للبيض.كما تم تلويث 20 بيضة خارجيا بالسالمونيلا انترتيدس ٨ x 10^5 خلية/بيضة وقسمت الى مجموعتين .المجموعة الاولى استخدامت كضابط ايجابى لميكروب السالمونيلا والمجموعة الثانية تم معالجتها بغاز الأوزون (٧٠ ميكرولتر / مل لمدة ٤٠ دقيقة) مما ادى الى انخفاض ميكروب السالمونيلا على سطح قشرة البيض بنسبة ٩.٥ لو / بيضة و تم عزل ميكروب السالمونيلا انترتيدس من ١٠٪ من العينات المعالجة.