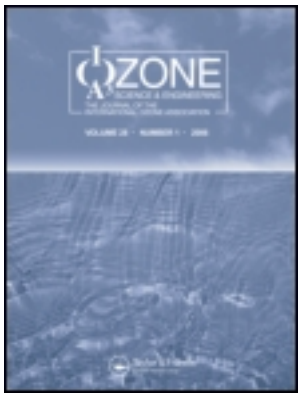

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Investigations on the Effect of Ozone as a Disinfectant of Egg Surfaces

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Investigations on the Effect of Ozone as a Disinfectant of Egg Surfaces

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The feasibility of gaseous ozone to reduce the number of microorganisms on the shell surface, of Salmonella Enteritidis (S.E.) in particular, of avian hatching eggs was investigated. Shell eggs were externally contaminated with S.E. 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 10^2–10^4 cfu 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.

Keywords Ozone, Ozone Treatment, Hatching Eggs, Disinfection, *Salmonella* Enteritidis

INTRODUCTION

Salmonellosis belongs to the most important zoonoses throughout the world. Current national statistical data indicate a recurring increase with 43,000 human infections in 2008 (personal communication, RKI, http://www3.rki.de/SurvStat/, 2009) Surveillance performed by the Enter-net National Reference Laboratories since 1993 has shown that *Salmonella* Enteritidis (*S*.E.) continues to be the predominant *Salmonella* serovar in Western Europe. Egg and egg products in particular are known as frequent sources of infection for consumers. Strong interest in methods to combat salmonellosis in poultry flocks exists for that reason. *Salmonella* spp. were found in 29.3% of large-scale German laying hen flocks (BfR, 2005). Studies of Cox et al. (1990) showed that in hatcheries, the floor or the conveyers were strongly contaminated with different serovars of salmonellae. Similar results were confirmed by Davies and Breslin (2003a) for egg packing facilities.

Less than 5 cfu/chick are sufficient to cause an infection (Milner and Shaffer, 1952). Therefore, formalin fumigation (usually a mixture of 42.4 ml formalin and 21 mg potassium permanganate per cubic meter room) has been used for disinfection of hatching eggs for many years. Dorn (1959) and Williams (1970) reported that this treatment reduces *Salmonella* spp. almost completely (99.8%) within 20 min. Usually, the eggs are treated shortly after laying to protect the blastodisk. In case that the regulatory authorities ban the use of formalin because of its carcinogenic and irritant properties, alternative methods ought to be available.

Ozone (O_3) is known as a highly reactive antimicrobial agent. According to the scientific literature, ozone treatment has been extensively tested for potential application in the food industry, i.e., decontaminating hatcheries, hatching eggs, and poultry carcasses. In 2001, FDA approved ozone as an antimicrobial for food. Besides the bactericidal effect, substantial advantages of ozone are its minor toxicity and easy handling. Because of the spontaneously decomposition into non-toxic-oxygen and readily biodegradable substances there are nearly no residues.

However, investigations demonstrating the effect of ozone on contaminated eggshells led to controversial results that might be due to the differing testing parameters. Koidis et al. (2000) obtained a slight bacterial reduction (1.19 $\log_{10}$) on the surface of eggs artificially contaminated with *S*.E. (initial dose 4.7 $\log_{10}$) using 1.4 mg ozone/L for 60 sec at 22 °C. Studies by Bailey et al. (1996) showed that after a treatment with 0.2–0.4ppm ozone 90.9% of the egg shells were still contaminated at the time of hatching.

On the contrary, recent studies of Rodriguez-Romo and Yousef (2005) demonstrated that a dose of 12–14% ozone wt/wt (generator) for 10 min results in a 5.9 $\log_{10}$ reduction.

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Whistler and Sheldon (1989a) reported a 2.5 $\log_{10}$ reduction of the natural bacterial count after an exposure to 3.03% ozone (wt/wt) for 2 h. Therefore, the objective of our project was to evaluate the efficacy of gaseous ozone, i.e., to develop an optimal protocol (dose-exposure time) to eliminate *S*.E. from contaminated egg shells while avoiding damages to the embryo. Methods for detecting changes in the nutritional composition of the egg (Fuhrmann et al., 2010) and embryonic damages after ozonation are/will be summarized in separate papers.

MATERIAL AND METHODS

To establish ozonation protocols, eggs from a local supermarket were used whereas for the hatching trials eggs of laying hen breeds (Lohmann White and Hyline Brown) were bought from a hatchery. To minimize effects of strain variabilities, three different *S*.E. strains phage-type 4 (provided by the Robert-Koch-Institute), isolated from egg shells, egg yolk and mixed egg content were used for this investigation. Egg surfaces were contaminated by dropping 0.1 mL of salmonella containing broth (incubated 37 °C for 24 h in nutrient broth (Sifin 1172), or diluted in log10 steps) on the shell to contain two contamination doses: 10^2–10^4 and 10^5–10^6 cfu/egg shell. After drying of two hours at room temperature, the eggs were placed in an ozone chamber with a capacity of 16.7 Liters, which allowed treating 48 eggs per trial. 10 out of 48 eggs (randomly selected) were bacteriologically examined.

Eggs were exposed to several ozone concentrations ranging from 0.5 to 5% wt/wt, in combination with relative humidity of < 30 or > 70% at an average temperature of 20 °C. After the desired concentration was reached, the eggs were left in the ozone chamber for a defined period of time (=exposure time). Exposure times varied between 20 min and 24 h. Then, 100 treatments were performed with eggs containing the higher contamination dose (n = 800 eggs) and 88 with the eggs containing the low dose (n = 780 eggs). The same number of contaminated, but untreated eggs were used as control group (n = 1580).

The ozone was generated by passing compressed oxygen through an ozone generator (BMT Messtechnik GmbH, Stahnsdorf, Germany), measured at the outlet of the chamber by an ozone analyzer (BMT 963). Concentration is expressed as percentage ozone by weight.

The enumeration of *S*.E. on the shells was done after shell contamination and after ozone treatment. Treated and control eggs were cracked aseptically, egg contents were discarded, and the shell of each egg was crushed. Shells were homogenized and washed with 0.9% NaCl solution (1:9).

Subsequently, aliquots of 0.1 mL of the serial decimal dilutions were plated on XLD (Xylose lysine deoxycholate) agar (Sifin TN 1196) and incubated for 24 h at 37 °C. Then colonies were counted. To exclude false negative results due to sublethal damages caused by the treatment and to retrieve vital salmonella, an enrichment was done by culturing the washing fluid in peptone water (Sifin TN 1137) and Rappaport-Vassiliadis medium (Sifin 1157) before plating it on XLD according to § 64 LFGB (formerly § 35 LMBG). Additionally, a sensory analysis (odor, taste, appearance) was performed by a trained panel of three scientists of the institute on the eggs from each treatment to test the suitability of ozone treatment for table eggs.

The data were obtained from five series of experimental runs. For statistical analysis, the results were processed using SPSS 11.5 (SPSS Software-GmbH München). One-way analysis of variance (ANOVA) and the Bonferroni test were performed to confirm differences among control and treatments at 95% confidence intervals. For a better comparability the results for the numbers of *S*.E. are shown on a logarithmic scale.

RESULTS

With our experiments we were able to prove that gaseous ozone reduces significantly *S*.E. on egg shells. Data in Table 1 demonstrate the important influence of the relative humidity during the ozone treatment. A significantly higher reduction ($p < 0.01$) of the number of salmonellae on the shell could be achieved using a relative humidity > 70%.

In Table 2 the instantaneous effect of three different ozone concentrations (0.5%, 2.5%, 5% wt/wt) without any exposure time is shown. After the desired concentration was reached in the ozone chamber, the generator was switched off. Though an ozone concentration of 5% resulted in a significant higher reduction of salmonellae, the maximum reduction was only 0.23%.

Therefore, exposure time has also to be considered as an important parameter. Different ozone concentrations, ranging from 0.3% to 5% wt/wt (0.3, 0.5, 0.7, 1.0, 1.5, 2.5, 3.0, 5.0), were evaluated at 6 exposure times (20, 40, 60, 90, 120, and 180 min), respectively. The results in Tables 3 and 4, give an example for the optimal dose-time combination to remove *S*.E. from the eggshell.

A dose of 1% (wt/wt) ozone for 120 min at a relative humidity of > 70% eliminated 10^2–10^4 cfu *S*.E. /egg shell completely. Even after enrichment no cells were recovered. Longer exposure times of up to 180 min did not result in a higher reduction rate. In hatching trials, resulting chickens were allocated to different keeping systems such as deep litter, cages and barns. Regular checks of the weight gain and the laying performance of the hens demonstrated that 1% ozone (wt/wt) for 120 min caused no negative effects. Higher ozone concentrations in combination with longer exposure times, as 3% for 180 min, resulted in a complete inactivation of *S*.E. on the shells as well; but DNA damages were detected.

Regarding the high contamination dose of 10^5–10^6cfu/egg shell, salmonellae were also reduced significantly up to approximately 6 $\log_{10,}$ as shown exemplarily in Table 5.

TABLE 1. Effect of Relative Humidity (rh) During Ozonation (0.5% wt/wt for 20 min) on the Reduction of *S*.E. (10^5–10^6cfu/shell)

rh	n	Control eggs ($\log_{10}$) Mean and SD	Ozonated eggs ($\log_{10}$) Mean and SD	Reduction ($\log_{10}$)
<30 %	30	5.95 ± 0.08	5.95 ± 0.08	0.04
>70 %	30	5.82 ± 0.09	5.13 ± 0.07	0.70*

*Significant reduction ($p < 0.01$).

TABLE 2. Instantaneous Effect of Ozone Concentrations at a Relative Humidity > 70% on the Reduction of *S*.E. (10^5–10^6cfu/shell)

Ozone (% wt/wt) /Exposure time	n	Control eggs ($\log_{10}$) Mean and SD	Ozonated eggs ($\log_{10}$) Mean and SD	Reduction ($\log_{10}$)
0.5%/0	10	5.59 ± 0.07	5.58 ± 0.04	0.00
2.5%/0	10	5.91 ± 0.10	5.87 ± 0.04	0.03
5.0%/0	10	6.23 ± 0.03	6.00 ± 0.04	0.23*

*Significant reduction ($p < 0.01$).

TABLE 3. Influence of Ozone Concentration for 120 min on the Reduction of *S*. E. (10^2–10^4cfu/shell)

Ozone concentration (% wt/wt)	n	Control eggs ($\log_{10}$) Mean and SD	Ozonated eggs ($\log_{10}$) Mean and SD	Reduction ($\log_{10}$)
0.3	50	2.94 ± 0.08	0.00 ± 0.00	2.94
0.5	50	2.99 ± 0.09	0.04 ± 0.04	2.95
0.7	50	3.84 ± 0.03	0.12 ± 0.07	3.72
1.0	50	3.28 ± 0.07	0.00 ± 0.00	3.28*

*No viable cells in enrichment.

TABLE 4. Influence of Exposure Time on the Reduction of *S*. E. (10^2–10^4cfu/shell) using 1% Ozone

Exposure time (min)	n	Control eggs ($\log_{10}$) Mean and SD	Ozonated eggs ($\log_{10}$) Mean and SD	Reduction ($\log_{10}$)M
60	50	3.07 ± 0.12	0.28 ± 0.16	2.79
90	50	3.68 ± 0.10	0.56 ± 0.19	3.12
120	50	3.28 ± 0.07	0.00 ± 0.00	3.28*
180	50	2.92 ± 0.10	0.00 ± 0.00	2.92*

*No viable cells in enrichment.

TABLE 5. Influence of Different Ozone Doses (0.5, 1, 3, 5%) and Exposure Times (120 or 180 min) on the Reduction of 10^5–10^6 *S*. E./Shell

Ozone (% wt/wt) /Exposure time (min)	n	Control eggs ($\log_{10}$) Mean and SD	Ozonated eggs ($\log_{10}$) Mean and SD	Reduction ($\log_{10}$)
0.5%/120	50	5.33 ± 0.04	0.21 ± 0.09	5.12
1.0%/120	50	5.26 ± 0.03	0.35 ± 0.11	4.91
3.0%/180	50	5.45 ± 0.10	0.00 ± 0.00	5.45
5.0%/180	50	6.23 ± 0.07	0.00 ± 0.00	6.23

However, up to 17% of the bacteria were damaged only sublethally by the ozone treatment (5% wt/wt for 180 min) and were recovered after enrichment.

Additionally, as described previously for the lower contamination dose, higher concentrations and/or a longer exposure times (e.g., 3% or 5% wt/wt ozone; 180 min) led also to satisfactory results regarding the elimination of *Salmonella* but egg nutrients as well as the DNA were affected significantly (Fuhrmann et al., 2010). Between the three tested *S*.E. strains, no significant differences were detected, regarding their sensitivity towards ozone.

Sensory testing of the ozonated eggs showed strong deviations in taste and smell which will evoke the consumers' disapproval. Therefore, it should be dissuaded from the ozonation of table eggs. Based on the results of the laboratory-scale tests an ozone cabinet (prototype) was developed, which holds 500 eggs. Its adequacy for the disinfection of hatching eggs was verified in an extensive testing phase, during which several improvements of the prototype were made.

DISCUSSION

A complete inactivation of 10^2–10^4 cfu *S*.E./egg shell, seeming relevant in practice, were reached by using ozone in a concentration of 1% (wt/wt) for 120 min. This is consistent with results of other research groups such as Whistler and Sheldon (1989a), Koidis et al. (2000) as well as Rodriguez-Romo and Yousef (2005). Besides dose and exposure time, the relative humidity is an important factor. For a bactericidal effect values should be > 70% confirming studies of Elford and van den Ende (1942), Foarde et al. (1997), and Davies and Breslin (2003b). According to Li and Wang (2003) the forming of free radicals at high relative humidities supports the oxidative effect of ozone.

Broadwater et al. (1973) described the effect of ozone as "all-or-none"-phenomenon, assuming that the inactivation will be complete after reaching a critical concentration. Other authors hypothesized a biphasic inactivation curve, starting with a fast inactivation which is followed by a slower (asymptotic) reduction of more resistant populations (Burleson et al., 1975; Katzenelson et al., 1979; Dyas et al., 1983; Foegeding, 1985; Herbold et al., 1989; Whistler and Sheldon, 1989b; Heindel et al., 1993; Restaino et al. 1995; Kowalski et al. 1998). Our findings confirmed the latter hypothesis.

The ozonation resulted in an early inactivation that increased linearly with ozone concentration in combination with longer exposure times. However, single *S*.E. cells were more resistant or could not be inactivated at all. While a concentration of 5–6 $\log_{10}$ *S*.E./egg shell could be reduced by about 5 $\log_{10}$ using ozonation with 1% ozone for 120 min, the lower contamination dose of 2.99 $\log_{10}$ on the shell was reduced by merely 2.95 $\log_{10}$. One reason might be the ineffectiveness of disinfections on bacteria hidden in the egg shell pores (Kuo et al., 1997); however, Rodriguez-Romo et al. (2007) found that ozone penetrates through the shell.

It can be concluded that gaseous ozonation seems suitable for applications in hatcheries. For practical use in the poultry industry, high-power ozone generators are necessary and the parameters (1% ozone for 120 min, > 70% relative humidity) should be verified in large ozone cabinets.

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