EFFECT OF FORMALDEHYDE TREATMENT ON BACTERIA-INFECTED HATCHING EGGS OF GALLUS GALLUS DOMESTICUS LINNAEUS, 1758

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ABSTRACT
The effectiveness of formaldehyde egg disinfection is well documented in literature despite its reported toxicity. This study focused on the need for an optimum formaldehyde concentration (FC) that significantly reduces microbial load with minimal damaging effect on egg viability and hatchability. Using a true experimental design, bacterial load on formaldehyde-treated (FT) and control groups of eggs and hatchability were compared. Gram-staining and biochemical tests identified five bacterial species: *Escherichia coli*, *Enterobacter* sp., *Bacillus cereus*, *Staphylococcus aureus* and *Micrococcus* sp. with the two coliform bacteria, *E. coli* and *Enterobacter* sp., dominating. Comparison of median differences of bacterial load on eggs before and after formaldehyde treatment by Wilcoxon Signed-Rank Test showed marginal significance in bacterial load reduction ($Z = -2.016, P = 0.044$). This difference was observed for bacterial load between the control group (CG) and the FT group with FC 30/20 ml/g ($U = 3.0, P = 0.047$). The hatchability of the CG differed significantly from four FT groups of eggs with FC 30/20 ml/g showing the highest level of significance [$\chi^2 (1) = 14.71; P = 0.0001$]. A FC of 30/20 ml/g produced the best domestic fowl egg disinfection compared to other FCs and hatchability decreased with increasing formalin volume.

Keywords: Formaldehyde treatment, bacteria-infected eggs, *Gallus domesticus*

Introduction
Microbial contamination of hatching eggs is a major issue in poultry production, as it can kill developing embryos, reduce hatchability and cause poor chick performance (Scott & Swetnam, 1993; Willinghan et al., 1996; Fasenko et al., 2009). At the right environmental conditions of soil, dust, dirty nesting materials (Abdul et al., 2012) and optimal conditions of temperature, nutrients and humidity (Graham et al., 2018), hatching eggs and chicks are at high risk of exposure to many species of microorganisms including bacteria and fungi (Oviasogie et al., 2016) and may ultimately lead to economic losses (Bailey et al., 1998; Kim et al., 2007). Some important groups of pathogens identified to be present in large amounts in hatch cabinets include *Escherichia coli*, *Pseudomonas*, *Staphylococcus*, *Salmonella* spp. and *Aspergillus* (Berrang et al., 1995), highlighting the importance of effective control of microbial contamination of eggs.

Fumigation of hatching eggs with formaldehyde has been described as one...
of the most effective methods of reducing bacterial load on eggs (Whistler & Sheldon, 1989; Kaudla, 1999; Yildirim et al., 2003; Cadirci, 2009), as it kills most viruses, bacteria (including their spores) and fungi (Salthammer et al., 2010; Swenberg et al., 2013). Although, formaldehyde is toxic and is reported to be carcinogenic to humans (USDHHS, 2010) and can be seriously damaging to embryo (Jasanoff, 1987; Hayretdag & Kolankaya, 2008), it is extensively used for microbial control within hatch cabinets (Cadirci, 2009; Kim & Kim, 2010) on account of its effectiveness. It is, therefore, imperative to find an optimum formaldehyde concentration that significantly reduces microbial load and at the same time have less damaging effects on egg viability and hatchability. This study assessed the effect of varied formaldehyde concentrations on bacterial load on *Gallus gallus domesticus* egg shell and hatchability.

**Experimental**

**Study area and sample collection**

The study was carried out at the Council for Scientific and Industrial Research (CSIR)-Animal Research Institute located in the Adenta Municipal District (5°42’25″N 0°10’15″W) in the Greater Accra Region of Ghana. Eggs (N=210), collected from the University of Ghana Farms, were transported to the hatchery unit, CSIR-Animal Research Institute and randomly allocated to 7 groups with 30 eggs per group.

**Sample preparation**

From each crate, six eggs were pulled to form a specimen and treated with different concentrations of fumigants before incubation in the hatchery (Bluestar Poultry Egg Incubator). Before fumigation, each egg forming the specimen was swabbed on the more blunted shell area with sterile microbiological swab (FL MEDICAL S.r.l. Tollegla, Italy) soaked with 0.1% blank peptone water (Merck, Darmstadt-Germany). The tip of the swab with the cotton bud was broken into MacCartney bottle, each containing 5ml of 0.1% blank peptone water to form the neat. This was done to know the microbial load before the fumigant was applied.

Two different concentrations of fumigants were prepared. In the first preparation, three different volumes of 40% formalin (30, 40 and 50) ml were added to 20g of potassium permanganate crystals each in a different container. In the second preparation, equal volume of formalin (40ml) was added to different amounts of potassium permanganate crystals (10, 15 and 25) g in different containers.

**Fumigation and incubation of egg**

Egg trays containing the egg samples were kept in separate air tight cardboard boxes of similar dimensions, 55 cm × 42 cm × 30 cm in length, breadth and height respectively. A metal container containing different concentration of the fumigant was put in each box and sealed immediately with cellotape. Samples were fumigated for 20min in a fumigation chamber and aired for 10min for all treatments with the exception of the control group.

Swabs were again taken after fumigation to form the second neat. The fumigated eggs were set for a 21-day incubation period at 37.7°C and 86% relative humidity prior to candling on the 18th day for egg fertility determination and 90% relative humidity after candling. Light penetration of a fertile egg revealed a distinct dark spot indicating embryonic development as well as blood vessels and air bubbles. However, infertile egg showed no distinct dark spots and egg content was clear, showing no signs of embryonic development. The specimen (bottles containing the neat),
before and after fumigation, were delivered on ice to the Microbiology laboratory of CSIR-
Animal Research Institute within four hours for laboratory investigation.

**Laboratory techniques**

**Specimen preparation**

One (1) ml of each neat was aseptically transferred into sterile screw cap MacCartney bottles each containing 9ml of 0.1% sterile blank peptone water [Merck, Darmstadt-Germany]. This was incubated at 37°C for 10-15minutes in a bacteriological incubator [Wagtech] (Collins *et al.*, 1995). Samples were serially diluted using 10-fold serial dilution into five others sterile MacCartney bottles containing 0.1% 9ml peptone water. Different pipette tips were used for each dilution.

**Culturing and total viable count (aerobic plate count)**

Media preparations and bacterial culture procedures were carried out as described by Heritage *et al.* (1996). For total viable count technique, the pour-plate method was used. One (1) ml of each dilution was aseptically added to 9ml of molten Standard Plate Count Agar [Merck, Darmstadt-Germany] kept at 45-50°C in a water bath [Grant, OLS 200]. This was mixed by rotation and poured into 9cm sterile Petri dish. It was allowed to cool, set and was incubated at 37°C for 24-48hrs. After incubation, plates showing between 30-300 colonies were selected and counted (Collins *et al.*, 1995) using electronic colony counter [Stuart Scientific]. Counts were derived by multiplying the colony(s) counted by the dilution factor. The counts obtained were expressed as x*10^y cfu/ml, where x is colony counted, 10^y is the dilution factor and cfu/ml as colony forming unit per millilitre.

**Total coliform count**

Using the plate-count technique, one (1) ml of each dilution was aseptically put into 9cm Petri dish. Nine (9) ml of molten Membrane Lactose Glucuronide Agar (MLGA) [Oxoid, CM 1031 Hamshire – England] kept at 45-50°C in a water bath (Collins *et al.*, 1995) was added, mixed by swirling and allowed to set. Plates were incubated at 37°C for 24-48hrs and examined for colonial morphology. Counts for each plate were multiplied by the dilution factor to obtain the number of colonies per sample.

**Faecal coliform count**

Using the plate-count technique, one (1) ml of each dilution was aseptically put into 9cm Petri dish. Nine (9) ml of molten Membrane Lactose Glucuronide Agar (MLGA) [Oxoid, CM 1031 Hamshire – England] kept at 45-50°C in a water bath was added, mixed by swirling and allowed to set. Plates were incubated at 42°C for 24-48hrs and examined for colonial morphology. Colonies showing greenish colour indicating faecal coliforms were selected and counted.

**Subculturing, isolation and identification of bacteria**

Impure cultures on primary media were purified by subculturing onto selected secondary media to obtain discrete colonies. Using a sterile inoculating loop, the neat samples were plated-out onto Blood Agar [Merck, Darmstadt-Germany] and Xylose Lysine Desoxycholate (XLD) agar [Oxoid, CM 0469, Hamshire – England] (Heritage *et al.*, 1996). Plates were incubated aerobically at 37°C for 24-48hrs in a bacteriological incubator (Wagtech). Cultures were examined for colonial characteristics on the media.
After overnight incubation, colonial morphology of organisms was studied for size, shape, outline, colour and change in medium on various media. Standard microbiological techniques including Gram staining were used to determine cellular morphology of organisms using compound microscope magnified at x100 with oil immersion. Organisms were isolated and identified using biochemical tests: Motility Indole Urea (MIU) [Lioflichems.r.l. Bacteriology Products, 610236, Italy], Catalase, Triple Sugar Iron (TSI) [Oxoid, CM 0277, Hampshire – England] and haemolysis. A well isolated colony was transferred onto the MIU agar with an inoculating needle for three test tubes. A loose cotton plug was placed over each test tube and incubated at 37°C for 18-24hrs to detect motility, urease enzyme activity and indole production ability of microbes. A small number of microbes was transferred using a sterile inoculating loop unto a microscopic slide devoid of any agar traces. A drop of 3% hydrogen peroxide was put onto the microbes on the slide using a dropper. Detection of bubbles indicates microbial production of catalase. To detect the haemolytic ability of microbes, a strain of a single colony was inoculated on blood agar and incubated at 35°C-37°C for 24hrs. Haemolytic activity was characterized by an absolutely or partially clear area around colonies. Non-haemolytic microbes were differentiated by a dark brownish coloration around a colony. To detect the reaction of microbes with glucose, sucrose, lactose and iron, a strain of a single colony was inoculated and streaked on the TSI agar slant. A loose cotton plug was placed over the tube and incubated at 35°C in ambient air for 18-24hrs.

Data analysis

Data analysis was carried out with the Statistical Package for Social Sciences (SPSS) software (20.0). Following normality check of the data, Wilcoxon Signed–Rank test was used to compare median differences of bacteria load before and after fumigation. The Mann Whitney-U test was used to compare the mean ranks of bacteria load for the control and treatment groups. Chi-squared test was used to assess percentage hatchability of egg samples. Mean bacterial load before and after formaldehyde treatment and their standard errors were compared (Fig. 1).

Results and discussion

Fertility and hatchability of eggs

Out of the 210 Lohmann Brown eggs used in the study, 107 (51%) were observed to be fertile, 58 (54.2%) of which were successful in hatching. Pairwise comparisons using Chi-square test revealed that hatchability of the control group differed significantly from four formaldehyde-treated groups of eggs with formaldehyde concentration (FC) 30/20 ml/g showing the highest level of significance \( \chi^2 (1) = 14.71; P = 0.0001, \text{Table 1} \).
### TABLE 1

*Formaldehyde treatment of G. g. domesticus eggs and their percentage hatchability.*

<table>
<thead>
<tr>
<th>CFTG (ml/g)</th>
<th>NIE (FE)</th>
<th>NEH (% ha.)</th>
<th>DP (95%CI)</th>
<th>χ² (df);</th>
<th>P-value (PC*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 00/00</td>
<td>10 (20)</td>
<td>3 (15.00)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30/20</td>
<td>12 (18)</td>
<td>14 (77.78)</td>
<td>62.78 (31.61, 79.21)</td>
<td>14.71 (1);</td>
<td>0.0001</td>
</tr>
<tr>
<td>40/20</td>
<td>12 (18)</td>
<td>12 (66.67)</td>
<td>51.67 (20.56, 71.32)</td>
<td>10.31 (1);</td>
<td>0.0013</td>
</tr>
<tr>
<td>50/20</td>
<td>12 (18)</td>
<td>11 (61.11)</td>
<td>46.11 (15.31, 67.10)</td>
<td>8.43 (1);</td>
<td>0.0037</td>
</tr>
<tr>
<td>40/10</td>
<td>16 (14)</td>
<td>4 (28.57)</td>
<td>13.57 (13.39, 41.42)</td>
<td>0.9 (1);</td>
<td>0.3427</td>
</tr>
<tr>
<td>40/15</td>
<td>17 (13)</td>
<td>7 (53.85)</td>
<td>38.85 (6.40, 63.79)</td>
<td>5.46 (1);</td>
<td>0.0195</td>
</tr>
<tr>
<td>40/25</td>
<td>14 (16)</td>
<td>7 (43.75)</td>
<td>28.75 (-0.73, 53.80)</td>
<td>3.56 (1);</td>
<td>0.0592</td>
</tr>
</tbody>
</table>

*Pairwise comparisons (PC) of control and formaldehyde treatment (ml/g) groups.

χ², Chi-square; CI, confidence interval. Hatchability of the control group differed significantly from four formaldehyde-treated groups of eggs.

Data are expressed as numbers, percentages (95%CI).

1CFTG: Control and formaldehyde treatment (ml/g) groups.

2NIE (FE): Number of infertile eggs (fertile eggs).

3NEH (%ha.): Number of eggs hatched (percentage hatchability).

4DP (95%CI): Difference in percentages (95%CI).

Although, formaldehyde disinfection of eggs is reported to improve upon hatchability (Shahein & Sedeek, 2014) in line with the findings of this study, many other factors are important. Fertility and hatchability of poultry eggs are influenced by many factors including diet (Brillard, 2007; Javanka *et al*., 2010), egg factors such as weight, shell thickness and porosity, shape index and the consistency of the content (Narushin & Romanov, 2002), hen age (Insco *et al*., 1947; Alsbayel, 1992) and optimum cock to hen ratio (King’Ori, 2011). Adequate diet in both quality and quantity is fundamental to production of good quality and number of eggs and semen (Brillard, 2007), which result in improved fertility and hatchability (Javanka *et al*., 2010). In particular, provision of fertile eggs with optimum environmental conditions including incubation temperature of 37.8°C (Lourens *et al*., 2007) or a range of 37.2°C and 37.7°C, egg turning (Yoshizaki & Saito, 2003; King’Ori, 2011) and relative humidity (60-80%) are fundamental to stimulating embryonic development until hatching (French, 1997). Under similar management practice and conditions of incubation, all other factors except disinfection status could be said to be similar in both control and treated groups of eggs following randomization. The significantly high percentage of hatchability in formaldehyde-treated groups of eggs highlights the importance of egg disinfection in poultry production.

**Disinfection of eggs**

Comparison of mean bacterial loads on eggs before and after formaldehyde fumigation showed that FC 30/20 ml/g appears to have
the best bacterial load reduction effect on the eggs (Fig. 1).

Using Gram-staining and biochemical tests, five bacterial species: *Escherichia coli*, *Enterobacter* sp., *Bacillus cereus*, *Staphylococcus aureus* and *Micrococcus* sp. were identified with the two coliform bacteria, *E. coli* and *Enterobacter* sp., dominating. Comparison of median differences of bacterial load on eggs before and after formaldehyde treatment by Wilcoxon Signed-Rank Test showed marginal significance in bacterial load reduction following formaldehyde egg treatment ($Z = -2.016, p = 0.044$). This difference was observed for bacterial load between the control group and the treated group with FC 30/20 ml/g ($U = 3.0, p = 0.047$).

Microbial contamination of eggs including *Escherichia coli*, *Bacillus cereus*, *Enterobacter* sp. (Oviasogie *et al.*, 2016), *Staphylococcus aureus* (McMullin, 2004), virulent *E. coli* (Chousalkar *et al.*, 2010), *Salmonella infantis* (Chousalkar *et al.*, 2013) present a lot of problems to commercial hatchery and its product, the chick. Within the first few minutes after lay, the eggshell is most ineffective barrier to bacterial invasion (Sparks, 1987). Under suitable conditions of moisture and temperature differential between the egg and the surrounding liquid (Lock *et al.*, 1992; Graham *et al.*, 2018), presence of shell microfractures (De Reu *et al.*, 2005), faecal contamination (Oviasogie *et al.*, 2016), among others, bacteria enter egg shell and its membranes. This could result in infection and killing of developing embryo, reduction in hatchability (Cadirci, 2009), spread of infection to hatched chicks through contact with contaminated eggshells and hatchery equipment (Cason *et al.*, 1994) as well as food safety concerns when human pathogens are involved. The marginally significant bacterial load reduction following formaldehyde egg treatment with 30/20 ml/g in this study appeared to have had an important positive impact on hatchability (Table 1), highlighting the importance of egg disinfection in poultry production.

**Conclusion**

The results of the present study suggest that disinfection of *Gallus gallus domesticus* eggs with formaldehyde concentration (FC) 30/20 ml/g results in highly significant improvement in hatchability. A FC of 30/20 ml/g produced the best domestic fowl egg disinfection compared to other FCs and hatchability decreased with increasing formalin volume. Therefore, the combination of egg disinfection with FC 30/20 ml/g and other relevant factors such as adequate diet in both quality and quantity, optimum cock to hen ratio, maintenance of optimum environmental hygiene and incubation conditions is expected to improve upon egg fertility and hatchability considerably.

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References


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