Microbial Load on Hatching Eggs from Farmhouse to Cold Room

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Abstract
This experiment was carried out to determine the concentration of microbial load, identify and isolate a specific microbe (E. coli) on hatching eggs from the breeder house, comparing it to subsequent storage durations in cold room. The study was conducted at the Department of Animal Science, K.N.U.S.T Kumasi, Ghana, where a total of thirty eggs were obtained from the poultry section and sent to the Microbiology Laboratory for the determination of microbes. The samples analyzed included fifteen eggs each from egg-laying nest boxes and floor of a deep litter housing covered with litter. Eggs were then stored in a cold room at 16oC and 75% RH for up to 12 days. Microbial sampling over the eggshell surfaces was conducted at various points of collection (breeder house) and 4, 8, as well as 12 days following storage. The sampling of bacteria from each treatment was done using swab sticks, which were then dipped into separate test tubes containing Peptone water. Samples were cultured on Nutrient agar for total viable bacteria population and MacConkey agar for E. coli. Both media used to grow bacteria were sterilized by autoclaving at a temperature of 121oC for 20 minutes before culturing. The culture was incubated at 37oC for 24 hours and total microbial count performed on all colonies identified. Citrate and Tryptone were used for a biochemical test for the identification of E. coli on the incubated samples. A colony counter was used for enumerating bacteria colonies. Data were analyzed using the GLM procedure of SAS at $P < 0.05$. Prevalence evaluation of the microbes showed that eggs collected from the floor had a high bacteria load as compared to eggs laid in the nest. The general bacteria load and E. coli load on the egg samples reduced when stored in the cold room and almost reached zero at 12 days. The E. coli population was higher also higher in floor eggs versus nest eggs. For food safety and reasons of chick quality, it is important that hatching eggs are stored appropriately prior to incubation and this will reduce bacteria multiplication, reduce the practice of washing dirty eggs before incubation which can affect chicks and increase post hatch chick mortality.

Keywords: Hatching eggs, breeder house, cold room, microbial load, E. coli

Charge Microbienne Sur Les Oeufs a Couver De La Ferme à La Chambre Frigorifique

Résumé
Cette expérience a été réalisée pour déterminer la concentration de charge microbienne, identifier et isoler un microbe spécifique (E. coli) sur les œufs à couver du reproducteur, en le comparant aux durées de stockage ultérieures en chambre frigorifique. L'étude a été menée au Département de zootechnie de K.N.U.S.T, Kumasi, au Ghana, où un total de trente œufs ont été obtenus de la section de la volaille et envoyés au laboratoire de Microbiologie pour la

détermination des microbes. Les échantillons analysés comprenaient quinze œufs provenant chacun de nichoirs de ponte et du sol d'un logement profond pour litière recouvert de litière. Des œufs ont ensuite été stockés dans une chambre frigorifique à 16 ° C et à 75% de HR pendant 12 jours maximum. Un échantillonnage microbien sur les surfaces de la coquille a été réalisé à divers points de collecte (élevage) et 4, 8, ainsi que 12 jours de stockage. L'échantillonnage des bactéries de chaque traitement a été effectué à l'aide de bâtonnets d'écouvillon, qui ont ensuite été plongés dans des éprouvettes séparées contenant de l'eau peptonée. Les échantillons ont été cultivés sur gélose Nutriment pour la population totale de bactéries viables et gélose MacConkey pour E. coli. Les deux milieux utilisés pour la croissance des bactéries ont été stérilisés par autoclavage à une température de 121°C pendant 20 minutes avant la mise en culture. La culture a été incubée à 37°C pendant 24 heures et la numération microbienne totale a été effectuée sur toutes les colonies identifiées. Le citrate et la tryptone ont été utilisés pour un test biochimique d'identification de E. coli sur les échantillons incubés. Un compteur de colonies a été utilisé pour dénombrer les colonies de bactéries. Les données ont été analysées en utilisant la procédure GLM de SAS à P <0,05. L'évaluation de la prévalence des microbes a montré que les œufs collectés au sol présentaient une charge bactérienne élevée par rapport aux œufs pondus dans le nid. La charge bactérienne générale et la charge de E. coli sur les échantillons d'œufs ont été réduites lorsqu'elles étaient stockées dans la chambre frigorifique et presque nulles au bout de 12 jours. La population de E. Coli était également plus élevée dans les œufs de sol que dans les œufs de nid. Pour des raisons de sécurité alimentaire et de qualité des poussins, il est important que les œufs à couver soient entreposés de manière appropriée avant l'incubation, ce qui réduira la multiplication des bactéries, réduira le lavage des œufs sales avant l'incubation, ce qui peut affecter les poussins et augmenter la mortalité post-éclosion.

Mots clés: œufs à couver, élevage, chambre frigorifique, charge microbienne, E. coli

Introduction

An egg is known for its rich protein source and other nutrients including calcium, vitamins, zinc, and acts as an antioxidant. Some of its constituents defend humans from many degenerative processes including cardio-vascular diseases. The defensive function is due to the presence of antimicrobial, immunomodulatory and anti-cancer compounds (Natoli et al., 2007, Samman et al., 2009, Fraeye et al., 2012). Egg value and production have evolved quickly and it is even predicted to further increase with the advancement in the management of poultry system. A study made by FAO estimated world egg production to increase by 30% in the year 2015. The figure was in contrast with the year 2000 value of egg production, with higher development rates in developing nations (FAO, 2003). There are two types of eggs: hatching eggs, used for incubation and production of day-old chicks, and table eggs which are produced for human consumption. However, all eggs are susceptible to some microbial infection but contamination on hatching eggs is very dangerous because it leads to infection on the embryo developing in the egg and reduces immunity in post-hatch day-old chicks (Berrang et al., 1999; Fasenko et al., 2009).

Microscopic organisms and mold, which can influence hatching eggs quality are present wherever there is earth - in the soil, in manure, and even on dust particles (USDA, 2012). The most common way hatching eggs get contaminated is by allowing laid eggs to lie on the floor or in dirty nests or and slats (van den Brand et al., 2016). At this point, a large number of bacteria can get onto the eggshell

surface. This increases the risk of bacteria attacking the inside of the egg (Holly, 2016). Microbes inside the egg may utilize the nutrients found in the egg to grow, robbing the embryo of a vital food source. They also produce toxins that are harmful to embryonic development and increases morbidity and mortality even in newly hatched day-old chicks (Hansen et al., 2015; Holly, 2016). Regardless of the possibility that the embryo of a contaminated egg may survive till hatching, the chick will either die in the broiler house or will have reduced growth, affecting the end of life productivity (Agulles, 2014). Contaminated eggs that do not hatch in the hatchery can likewise influence other healthy eggs. If one contaminated egg breaks in the incubator, it might spread bacteria to different eggs or newly hatched chicks, thus, contaminating the entire hatchery (Lucore, 1994).

Microbial contamination of eggs is common with enteric bacteria with Salmonella enteritidis being the greatest threat. Egg contents are suitable media for bacterial growth. Hence, the risk of egg contamination by pathogenic bacteria, especially S. enteritidis, is a major worry for egg production and egg product manufacturing industries (Baron and Jan, 2011). Measures taken to prevent contamination of the hens include breeding stocks for disease resistance, applying housing management systems and techniques that prevent cracked eggs. These have been implemented in the poultry industry to reduce microbial entry into eggs. Direct managerial strategies to reduce the spread of bacteria include to decontaminate facilities between flocks, vaccinate hens against pathogens, use pathogen-free feeds and feedstuffs, maintaining pest-free facilities, facilitating gastric microbiota development using probiotics and prebiotics to enhance passive immunity, ensuring clean facilities and maximizing biosecurity (Ruxton, 2013). The measures to keep pathogen-free eggs include collecting clean, unsoiled eggs, cooling eggs as soon as possible and maintaining them in cool, clean storage facility and pasteurizing contaminated eggs where possible (USDA, 2013). The objective of this study was to determine the microbial load on hatching eggs from the farm house through to storage up to 12 days of storage.

Materials and Methods

Experimental Materials and Design

Experimental samples were collected from the Poultry section of the Department of Animal Science, K.N.U.S.T- Kumasi, Ghana. A total of thirty eggs were collected from deep litter housing system. These included fifteen eggs from each of the nest boxes and floor covered by litter. Fresh eggs collected from the poultry pens from the floor and nest boxes were swabbed using wet swab sticks dipped into Peptone water. The swab sticks were wet with peptone water to ensure a maximum capture of the bacteria present on the eggshell surface. Each swab stick was then placed inside a test tube containing Peptone water and transported to the laboratory in an Ice chest containing ice blocks. The test tubes were labeled according to their respective contents and treatments, thus; eggs taken from the nest and those taken from the floor with litter. Each treatment contained 15 eggs and each egg served as an experimental unit of replications. The eggs were subsequently placed in cold room facility at 16oC and 75% relative humidity for various days of storage (4, 8 and 12 days) prior to further microbial analysis.

Media Preparation

Nutrient agar and MacConkey agar, Peptone water, Tryptone, Citrate were strictly prepared according to the manufacturer's instructions. All the media were sterilized by autoclaving at a temperature of 121^{0}C for 20

minutes. Sterilization was done to prevent contamination of media.

Isolation of Bacteria from Eggs

Samples were taken and kept in 30 test tubes each containing 5 ml of peptone water. Test tubes containing 9 ml of distilled water were used in the preparation of serial dilution from 10-1 up to 10-4 (Figure 1). Hot air oven was used to sterilize Petri dishes. The spread and pour method of bacteria culturing and enumeration was used to grow bacteria on nutrient agar as described previously (Sanders, 2012). For the MacConkey agar, swab sticks from the stock solution were rubbed on the surface of the agar. Both the MacConkey agar plates and Nutrients agar plates were incubated at a temperature of 37 C for 24 hours.

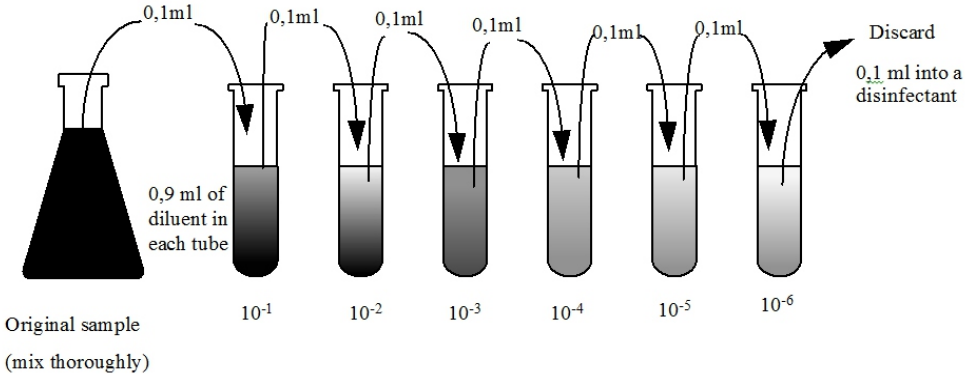
Colony counter was used to quantify the number of colonies that grew on the plates after incubation. A range of 30-300 was chosen for enumeration; samples below 30 and above 300 were too few or too numerous to count, respectively (TNTC). Calculation of the number of colony forming units (CFU) when using the spread and pour plate methods was performed as follows. The number of CFU/ml = N x 10n x 10; Where N = no. of colonies on the plate at the selected dilution n.

Identification of organism (E. coli)

In order to enumerate E. coli, the organisms were cultured on a selective media (MacConkey). It was later sub-cultured on nutrient agar and further cultured in peptone water. A biochemical test was done, using citrate, Tryptone, and Indole test to identify E. coli. The Indole reacts with Kovac's reagent to produce a cherry red complex, an indication that the organism was present. Samples were also cultured in citrate which is blue in color. The solution will remain blue if there is no organism (E. coli) present. But if E. coli is present it will change to green. Colonies counted by the colony counter were quantified by the range of 30-300. When the colonies counted were below 30 that was taken to be too small to obtain a viable count but when it exceeded 300 it was considered too numerous to count. A selective media (MacConkey agar) was used to make the enumeration of isolated sample easier.

Statistical analysis

Data were analyzed using the GLM procedure of SAS at $P < 0.05$. LS?? means were



separated using the SNK test (SAS, 2012). The fixed effects of place of egg collection and storage duration were analyzed for the level of bacteria on eggshells at the point of collection and during cold room egg storage for 4, 8 and 12 days.

Results and Discussions

B*acteria Load on Hatching Eggs from Breeder House to Cold Room*

Table 1 shows the bacteria load on hatching eggs at the various stages of egg collection and storage during the study. Eggs taken from the deep litter had high bacteria load on the eggshell surface compared to eggs from the nest. This could be attributed to the presence of fecal matter on litter. Eggs taken from the nest had relatively fewer bacteria load as compared to that of the floor eggs for all the days (day 4, day 8 and day 12). Generally, the eggs stored in the cold room had fewer bacteria load than the eggs kept at the ambient temperature.

When nest eggs were stored in the cold room, the bacteria count appeared to decrease as the eggs spent time in the breeder house. But the number of bacteria decreased when eggs were stored for 4 days in the cold room. However, from breeder house to 4 days of storage in cold room the bacteria load increased for eggs obtained from the floor of the poultry house. When eggs were stored for 8 days, the decrease in bacterial load was much lower in eggs obtained from next boxes while the bacteria load from the floor decreased much higher. When the eggs continued into cold room storage until 12 days the bacteria load decreased in both cases but the bacteria load was much higher in floor eggs compared to nest box eggs. The bacteria load in floor eggs tended to be significantly higher than those

Table 1. Total viable bacteria on hatching eggs surface area

Bacteria Load	Treatments/ Conditions			
	Breeder house	Cold Room (Day 4)	Cold Room (Day 8)	Cold Room (Day 12)
Nest eggs (CFU/ml)^{3}	9.88×10^{5}	4.52×10^{5}	4.44×10^{53}	5.97×10^{3}
Floor eggs (CFU/ml)	2.27×10^{5}	1.16×10^{63}	7.40×10^{5}	9.01×10^{3}
SEM	4.62×10^{5}	8.53×10^{5}	6.49×10^{5}	16×10^{3}
P-value		0.258	0.555	0.079

Table 2. Identification of E. coli on eggshell surfaces in four conditions of egg storage

Condition of storage	E. coli load of egg samples from the nest (T_1) (CFU/ml)	E. coli load of egg samples from the floor (T_2) (CFU/ml)	P values
Breeder house	6.60×10^{6}	8.88×10^{6}	0.5299
Cold room (day 4)	5.31×10^{6}	8.82×10^{6}	0.1469
Cold room (day 8)	9.19×10^{7}	6.85×10^{6}	0.3807
Cold room (day 12)-	No Growth^{7}-	1.22×10^{7}-	1.22×10^{7}-

from nest boxes ($P < 0.079$). It appears that higher bacteria load on the eggshell surface from the point of collection till the end of storage may not be influenced to a greater extent by cold storage. Since the bacteria load from floor eggs started as high and also ended as high, floor eggs may not be appropriate for setting in incubators as this may affect egg viability and embryonic development (Berrang et al., 1999; Cook et al., 2005; Fasenko et al., 2009).

This study has shown that microbes on eggshells of newly laid eggs can increase quickly when exposed to ambient conditions (Jones et al., 2004). A higher microbial load can increase penetration from the eggshell surface area through the pores, and lead to dramatic reduction in hatching performance (Berrang et al., 1999). According to studies conducted by FAO in 2004, it was noted that proper egg storage and handling at a temperature between 10 and 15˚C significantly contributed to preserving the quality of hatching eggs.

The floor eggs appeared to record a higher E. coli load than those from the nest eggs. In the nest eggs, the bacteria levels increased as the eggs moved from the breeder house up to 8 days of cold room storage. At 12 days of storage, no E. coli growth was recorded in the nest eggs. In the floor eggs, bacteria population reduced as the eggs moved from one condition of storage to other. The study shows that extensive multiplication of S. enteritidis was less frequently observed at lower inoculum doses (15 cells), shorter storage times (1 day), and lower temperatures (10 to 17.5oC) and when contaminants were introduced into the albumen (Gast and Holt, 2000). This study appears to emphasize the need to store cold temperatures eggs to reduce bacteria growth.

It was expected that the E. coli population will be lower than the total bacteria load (Table 1). Surprisingly, this was not the case in the current study. It is likely the differences in the growth of bacteria were due to the different media used in culturing the samples to enumerate total bacteria and E. coli alone in another instance. It is well supported in the literature that the nutrient agar is used to enumerate total bacteria population which supports both gram-negative and gram-positive bacteria. Also, the MacConkey agar supports the growth of only gram-negative bacteria, which E. coli is a member. It may appear that on the nutrient agar there was competition between different bacteria to grow which was not the case for E. coli on the MacConkey agar where it freely multiplied. The nutrient agar was also not the best media to support faster bacteria growth.

One other reason for the higher growth of the E. coli than the total bacteria count was that in all cases the studied looked for viable bacteria count. For the E. coli, serial dilutions up to level 4 had to be made to find viable counts. However, to obtain the total viable bacteria count on nutrient agar only the first and second solutions were enough to see viable bacteria count on the nutrient agar. Since the samples were largely fecal materials or litter infested, the appearance of gram-negative bacteria such as E. coli was likely. However, it is not certain why other bacteria and the E. coli did not grow very well on the nutrient agar. That has to be investigated in future experiments. Nevertheless, the media required different serial dilutions to obtain bacteria viable count which could account for the variation in the numbers. The differences in growth are support by previous research, which show that growth potential of bacteria isolates on MacConkey agar recorded the highest growth potential of 8.9 x 105 CFU/ ml for E. coli followed by Blood Agar that gave 8.8 x 105 CFU/ml for Shigella. The third highest growth potential of 8.6 x 105 CFU/ ml

was recorded in nutrient agar against S. aureus (Ifeanyi et al., 2014).

Previous studies have revealed that poor hygienic conditions in poultry houses predispose eggs to contamination by fungal, viral and bacterial pathogens especially Enterobacteriaceae such as E. coli (Poopes, 2000). According to Al-khalaf, et al. (2010) the importance of E. coli contamination of eggshells and hatchery losses cannot be overlooked. Awaad (1972) reported that E. coli could penetrate the eggshell inducing high embryonic mortalities. The outcome of this study shows that keeping eggs in the cold room for a relatively long period reduced the E. coli population. However, there was no significant difference in E. coli population between nest or floors eggs. It is worth to note that although the differences were not significantly different between nest eggs and floor eggs, there may be biological implications of higher bacteria population. A dirty egg can have a serious impact on incubation results. This is practically the case in floor eggs, which often explode during incubation. Hatcheries, therefore, wash dirty eggs in warm water but this can reduce hatchability because the practice leads to damaged eggshell ultrastructural features such as cap quality, alignment, causes erosion and confluence, kills Type B bodies and removes cuticle cover, as well as creating entry for bacteria to infest embryos before they hatch (Gole et al., 2014).

The relatively higher microbial load on the floor eggs as compared to the nest eggs can be attributed to dirty environment and can be worse with old litter. This leads to poor hatchery practice of washing eggs before incubation which removes the cuticle and expose embryos to contamination during incubation (Board and Halls, 1973). Also, inefficient and ineffective management systems or cleaning in poultry farms leads to the presence of fecal matter on the litter. Higenyi and Kabasa (2014) mentioned that the intensive system exposes poultry and poultry products to heavy bacterial contamination loads. This is consistent with another study which found that intensive management predisposes poultry to bacterial infections leading to major health problems in poultry flocks (Foley et al., 2008). The lack of laying nests on farms for birds to lay eggs or early placing the nest in laying pens before hens start laying lead to higher number of floor eggs. For some birds, it is a bad laying behavior and it is breed related (Campo et al., 2007). These factors could be contributing to the increased total bacteria load recorded in this study.

The study underlines that cold storage of hatching eggs below physiological zero irrespective of the source of eggs is important before incubation to reduce bacteria load and this could reduce the use of chemicals or fumigation on eggs, further emphasizing food safety standards that should start from the hatchery. Proper cold storage and taking note of duration of storage could reduce the practice of washing dirty eggs before incubation which removes the cuticle. The current study, though with small sample size has demonstrated the peculiar importance of microbes on eggs which could have much consequence on hatchery performance and contamination

In conclusion, eggs collected from the floor had high bacteria load than that of eggs laid in the nest, while cold room storage for day 12 reduced the bacteria load as compared to the breeder house, cold room eggs at days 4 and 8. The study also showed that E. coli load on the egg samples reduced when stored in the cold room. Therefore, hatcheries need to put in place proper biosecurity to receive less dirty eggs.

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