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**Research Article**

# COMPARATIVE BACTERIAL CONTAMINATION OF EGGS RAISED UNDER DIFFERENT MANAGEMENT SYSTEMS IN PUNJAB, INDIA

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## SUMMARY

A cross-sectional study was designed to investigate differences in contamination rates of eggs surface from the deep litter and caged poultry layers farms across five districts in Punjab, India. Around 24 farms (12 per category) were randomly selected and farmers were surveyed using a well-informed questionnaire. Additionally, a total of 480 freshly laid eggs were aseptically collected and subjected to estimation of total bacterial count (TBC), total Enterobacteriaceae count (TEC) and occurrence of *S. aureus* and *E. coli* (critical pathogens). The analysis revealed that deep litter system harbored eggs with significantly higher TBC, TEC, *S. aureus* and *E. coli* compared to cage system. The association of occurrence of the *S. aureus* and *E. coli* from different systems with respect to the factors present on the farm was confirmed using generalized linear model (GLM) to associate self-formulation of feed and absence of biosecurity with higher occurrence of *S. aureus* and *E. coli* in deep litter farms. Therefore, the study highlights the need for implementation of strict biosecurity and sanitary measures during feed formulation for birds and egg production at farm level to minimize the chances of transmission of bacterial load into the consumer's food chain.

**Keywords:** Cage system, Deep litter, Egg, *Escherichia coli*, *Staphylococcus aureus*

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The presence of all essential amino acids (AAs) and non-essential AAs makes poultry eggs the most nutritious foods of animal origin (Kralik *et al.*, 2017). At the time of production, the microbiological safety of eggs is attributed to the innate protection provided by eggshell and a semi-permeable membrane, however, the risk of contamination mainly bacterial in nature upsurges after laying due to the contact with environmental contaminants. Furthermore, the migration of these surface contaminants inside the egg is facilitated by improper handling and unhygienic storage of eggs (high moisture and temperature) (Pyzik and Marek, 2012). Apart from the spoilage causing organisms, a number of clinically significant and food-borne bacterial species such as *Salmonella enterica*, *Yersinia enterocolitica*, *Listeria monocytogenes*, methicillin resistant *Staphylococcus aureus* and *Escherichia coli* have been previously reported from table eggs (Syed *et al.*, 2018). While the surface bacteria pose a major threat to consumer's health, only limited surface decontamination procedures for the eggs are in use worldwide, including countries like United States of America (USA), Australia and Japan (Hutchison *et al.*, 2004). However, few studies have previously reported the inefficiency of current sanitization techniques employed in the commercial egg industry (Al-Ajeeli 2017). Therefore, it can be presumed that bacterial strains with relatively higher tolerance to disinfectants have higher chances of survival on the eggshell surface and are more likely to enter the food chain. Furthermore, very limited studies have been conducted exploring the differences in surface contamination over the egg surface with respect to the systems of poultry bird rearing. Therefore, present study was undertaken to examine the difference in contamination rate and occurrence of priority pathogens i.e., *S. aureus* and *E. coli* on eggs raised under deep litter and cage system.

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## MATERIALS AND METHODS

### Survey

A meticulously crafted questionnaire was validated through farmer participation at the Kisan Mela, before its subsequent deployment at selected farm sites. The questionnaire was so designed to assess the distinctive characteristics and disparities in management systems and agricultural practices between deep litter and cage farms.

### Sample collection and processing

The bacterial contamination was expressed as total bacterial count (TBC) and total *Enterobacteriaceae* count (TEC) per unit surface area ($mm^2$) of eggshell. 24 poultry layer farms (12 per category) across five districts of Punjab *viz*., Ludhiana, Amritsar, Bhatinda, Rupnagar and Mansa were sampled using convenient and purposive sampling technique representing different geographical regions in the state of Punjab. Further, a total of 20 freshly laid eggs were collected from each farm, thus making it to a total of 480 egg samples.

The processing of eggshell and inner content was

followed using method described by Musgrove *et al.* (2005). Initially, each egg was aseptically handled to measure length and breadth using digital vernier calipers (Advance, Mumbai), followed by cracking of egg at air-sac end using sterile glass rod. The inner content was separated and collected in sterile disposable sampling bag in aseptic manner. The remaining eggshell was put in a sterile disposable bag containing 20 ml of buffered peptone water (BPW) and hand crushed before estimation of TBC, TEC and isolation of *Staphylococcus aureus* and *Escherichia coli*, potentially present on the eggshell. The separated inner content of each individual egg was only used for the isolation of *S. aureus* and *E. coli*.

**Total bacterial count (TBC) and total *Enterobacteriaceae* count (TEC)**

For the determination of total bacterial count (TBC), each eggshell was washed with 20 ml of BPW in a sterile sampling bag (Nasco sampling bag, HiMedia Lab, Mumbai); 10 ml of wash was used for performing TBC. The wash of 5 eggs was pooled to make one lot. Four such lots were used for each farm to estimate TBC. The formulae used for calculation of surface area of egg and estimation of TBC are mentioned ahead (Equations 1-3). For TBC, after dilution of egg wash, 0.1 ml of the sample was spread over the plate count agar (HiMedia lab, Mumbai) and inoculum was spread evenly using 'L' shaped disposable spreader. The plates were incubated at 37º C for 24-48 h. After incubation, the plates depicting colonies ranging from 25-250 were selected and the colonies were counted using colony counter.

**Calculation:**

**Equation 1:**

$$\text{TBC or TEC/surface area of eggs } (mm^2) = \frac{\text{TBC (CFU) or TEC of eggshells from farm}}{\text{Total surface area of eggshells } (mm^2)}$$

**Equation 2:**

Surface area of the eggshell (S.A) ($mm^2$) = (3.155-0.0136L + 0.0115B) L x B; L= length (mm), B= breadth (mm) (Narushin and Romanov, 2002)

**Equation 3:** Colony forming unit (CFU/ml) = Average no. of colonies × reciprocal of dilution factor

For determination of TEC, 1 ml of diluted samples was poured on sterile petriplates in duplicate. Later, 15 ml of molten violet red bile agar (VRBA), priorly equilibrated at 50º C in water bath was poured over the sample and swirled to mix it with the agar. The agar was allowed to solidify and overlaid with 10-15 ml of VRBA followed by incubation at 37º C for 24 h. The purple red colonies with diameter equal to or more than 0.5 mm were suggestive of members of *Enterobacteriaceae*. The plate depicting colonies not more than 150 were selected and TEC was determined using the formula mentioned in equations 1-3.

The remaining 10 ml of the individual eggshell wash was used for isolation of *S. aureus* and *E. coli*.

**Isolation and identification of *S. aureus* and *E. coli***

Egg samples were processed for *S. aureus* isolation following a method outlined by Syed *et al.* (2018). Egg shell wash and inner content were enriched in trypticase soy broth (TSB) with 10% NaCl and sodium pyruvate and then incubated overnight. A loopful from each sample was streaked onto Baird Parker agar (BPA) with egg yolk telurite emulsion and incubated. Typical colonies were picked, streaked onto brain heart infusion (BHI) agar for purification, and subjected to Gram's staining and catalase test. Positive colonies, showing characteristic morphology and catalase reaction, were stored in BHI broth with glycerol at -20º C.

The isolation of *E. coli* from eggs was performed using method described by Mezhoud *et al.* (2016). The eggshell wash and the inner content were enriched in BHI broth prior to selective plating on eosin methylene blue (EMB) agar. The presumptive colonies producing metallic sheen were further confirmed using matrix assisted laser desorption time of flight (MALDI-TOF).

**Molecular confirmation of *Staphylococcus aureus***

The presumptive *S. aureus* were confirmed using genus and species specific polymerase chain reaction (PCR), while ATCC 25923 *S. aureus* strain was used as the positive control. The cycling conditions and primer details have been tabulated in table I.

**MALDI-TOF**

Presumptive *E. coli* colonies were cultured on BHI agar plates and incubated at 37º C for 24 h. 0.1 mg of cell material from each isolate was transferred to a MALDI target site, treated with 70% formic acid and matrix solution (10 mg/mL concentration of -cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and then analyzed. Scores ranging from 1.7 to 2.3 indicated varying levels of genus and species identification reliability, with scores above 2.3 indicating highly probable species identification.

**Statistical analysis**

The statistical analysis was conducted using IBM SPSS Statistics v.26.0 (IBM Corporation, Armonk, NY). The normality of data distribution (TBC and TEC) was checked using Kolmogorov-Smirnov test and the comparison of means between different management systems was done using Kruskal Wallis (TBC) and independent 't' test (TEC),

**Table 1. Primers used for the confirmation of *S. aureus***

| Category | Gene | Primer sequence | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| Genus specific | 16S RNA | F: CAGCTCGTGTCGTGAGATGTR: AATCATTTG TCCCACCTTCG | 420 | (Strommenger *et al.*, 2003) |
| Species specific | nuc | F: GCGATTGATGGTGATACGGTTR:AGCCAAGC CTTGACGAACTAAAGC | 279 | (Brakstad *et al.*, 1992) |

**Table 2. Farm characteristics of layer poultry farms based on different system of rearing**

| Variables | Response | Frequency of farms | | |
|---|---|---|---|---|
| | | Deep litter (N=12) | Cage (N=12) | Total (N=24) |
| Gender | Male | 12 (100%) | 12 (100%) | 24 (100%) |
| | Female | 0 | 0 | 0 |
| Type of farming | Only poultry | 10 (83%) | 12 (100%) | 22 (91.6%) |
| | Mixed | 02 (16.6%) | 0 | 02 (8.3%) |
| Feed purchase | Self-formulated | 10 (83.3%) | 0 | 10 (41.6%) |
| | Commercially purchased | 02 (16.6%) | 12 (100%) | 14 (58.3%) |
| Any cleaning of eggs practiced | Yes | 0 | 02 (16.6%) | 02 (8.3%) |
| | No | 12 (100%) | 10 (83.3%) | 22 (91.6%) |
| Use of litter as manure | Yes | 12 (100%) | 12 (100%) | 24 (100%) |
| | No | 0 | 0 | 0 |
| Farm biosecurity | Present | 05 (41.6%) | 12 (100%) | 17 (70.8%) |
| | Absent | 07 (58.3%) | 0 | 07 (29.1%) |
| Commonly reported diseases | Chronic respiratory disease (CRD) | 08 (66.6%) | 02 (16.6%) | 10 (41.6%) |
| | Gumboro | 05 (51.6%) | 0 | 05 (20.8%) |
| | Coccidiosis | 10 (83.3%) | 02 (16.6%) | 12 (50%) |
| | Vitamin deficiency | 10 (83.3%) | 0 | 10 (41.6%) |

**Table 3. Total bacterial count on eggshells from different systems of poultry rearing**

| System of farming | | | |
|---|---|---|---|
| Deep litter | | Cage farms | |
| Farm ID | Average $\log_{10}$ CFU/mm$^2$ | Farm ID | Average $\log_{10}$ CFU/mm$^2$ |
| LD-1 | 6.39 | AC-1 | 5.21 |
| LD-2 | 5.06 | AC-2 | 3.92 |
| LD-3 | 4.69 | AC-3 | 3.87 |
| LD-4 | 5.02 | AC-4 | 5.13 |
| MD-1 | 5.0 | BC-1 | 3.69 |
| MD-2 | 5.11 | BC-2 | 4.95 |
| MD-3 | 5.34 | BC-3 | 3.90 |
| MD-4 | 5.92 | BC-4 | 3.87 |
| RD-1 | 5.32 | LC-1 | 4.02 |
| RD-2 | 6.29 | LC-2 | 4.09 |
| RD-3 | 5.38 | LC-3 | 3.95 |
| RD-4 | 5.37 | LC-4 | 5.32 |
| Mean $\log_{10}$ CFU/mm$^2$±S.E | $^a$5.40±0.152 | $^b$4.32±0.179 | |

a,b different superscripts denote significant difference in between two different farming systems ($p<0.05$)

**Table 4. Total Enterobacteriaceae count per unit (mm$^2$) eggshell surface from different systems of poultry rearing**

| System of farming | | | |
|---|---|---|---|
| Deep litter | | Cage farms | |
| Farm ID | Average $\log_{10}$ CFU/mm$^2$ | Farm ID | Average $\log_{10}$ CFU/mm$^2$ |
| LD-1 | 2.65 | AC-1 | 2.53 |
| LD-2 | 2.70 | AC-2 | 2.56 |
| LD-3 | 2.78 | AC-3 | 2.58 |
| LD-4 | 2.69 | AC-4 | 2.31 |
| MD-1 | 2.73 | BC-1 | 2.39 |
| MD-2 | 2.80 | BC-2 | 2.35 |
| MD-3 | 2.74 | BC-3 | 2.47 |
| MD-4 | 2.67 | BC-4 | 2.45 |
| RD-1 | 2.68 | LC-1 | 2.46 |
| RD-2 | 2.68 | LC-2 | 2.58 |
| RD-3 | 2.75 | LC-3 | 2.41 |
| RD-4 | 2.69 | LC-4 | 2.51 |
| Mean $\log_{10}$ CFU/mm$^2$±S.E | $^a$2.71±0.013 S.E | $^b$2.46+0.025 S.E | |

a,b different superscripts denote significant difference in between two different farming systems ($p<0.05$)

**Table 5. *Staphylococcus aureus* contamination of egg samples collected from different systems of poultry rearing**

| System of farming | Sample | Sample size | Sample (+) for *S. aureus* | Occurrence % | Overall occurrence % |
|---|---|---|---|---|---|
| Deep litter | Eggshell | 240 | 37 | 15.4 | [a]30 |
| | Inner content | | 35 | 14.5 | |
| Cage | Eggshell | 240 | 39 | [a]16.25 | [b]20 |
| | Inner content | | 09 | [b]3.75 | |

a, b different superscripts indicate significant difference in *S. aureus* contamination in sample from different farming system ($p<0.05$).

**Table 6. Generalized linear model (GLM) estimates and odds ratio for occurrence of *S. aureus* with the predictor variables**

| Predictor | Odds ratio | p value | 95% Wald Confidence interval |
|---|---|---|---|
| Farm type (Deep litter) | 13.815 | 0.000 | 1.826 to 3.425 |
| Feed formulation (self-formulated) | 0.198 | 0.000 | -2.46 to -.775 |
| Biosecurity (present) | 0.295 | 0.000 | -1.894 to -0.544 |
| Medication (self-prescribed) | 0.992 | 0.000 | -0.612 to 0.596 |

respectively. The results were expressed as significant if $p<0.05$.

To analyze the association of response of farmers and contamination percentage of *S. aureus* and *E. coli* in different management systems, generalized linear model (GLM) was employed; wherein farm type, feed formulation, biosecurity were taken as predictors and contamination of *S. aureus* and *E. coli* was taken as dependent variable. To model the occurrence of *S. aureus* and *E. coli*, binomial family distribution with logit function (link = "logit") was used. The difference in occurrence of *S. aureus* and *E. coli* between the management systems was assessed using chi-square statistic, with $p<0.05$ taken as significant.

## RESULTS AND DISCUSSION

### Survey

Of all the characteristics surveyed, the major difference in between the two management systems were reportedly; (i) self-formulated feeds were utilized by majority {83.3% (10/12)} of the deep litter farms, while all the caged farms used commercialized feed (12/12; 100%) (ii) None of the deep litter farms engaged in cleaning of eggs, however only 2 out of 12 (16.6%) caged farms practiced cleaning of soiled eggs (iii) With respect to farm biosecurity; 41.6% (5/12) of the deep litter farms and all the caged farms allowed only authorized farm workers to enter inside the farms.

**Table 7. *Escherichia coli* contamination of egg samples collected from different system of poultry rearing**

| System of farming | Sample | Sample size | Sample (+) for *S. aureus* | Occurrence % | Overall occurrence % |
|---|---|---|---|---|---|
| Deep litter | Eggshell | 240 | 59 | [a]24.5% | [a]31.25 |
| | Inner content | | 16 | 6.6% | |
| Cage | Eggshell | 240 | 34 | [b]14.1% | [b]18.75 |
| | Inner content | | 11 | 4.5% | |

a,b different superscripts indicate significant difference in *E. coli* contamination in sample from different farming system ($p<0.05$).

**Table 8. Generalized linear model (GLM) estimates and odds ratio for occurrence of *E. coli* with the predictor variables**

| Predictor | 95% Wald Confidence interval | p value | Odds ratio |
|---|---|---|---|
| Farm type (Deep litter) | 0.588 to 0.5318 | 0.03 | 1.769 |
| Feed formulation (self-formulated) | 0.104 to 1.089 | 0.069 | 0.337 |
| Biosecurity (present) | 0.215 to 0.850 | 0.015 | 0.427 |
| Medication (self-prescribed) | 0.866 to 2.771 | 0.140 | 1.549 |

### TBC and TEC

The TBC of eggshell composite lots ranged from 5 $\log_{10}$ to 6.39 $\log_{10}$ in deep litter farms and from 3.69 $\log_{10}$ to 5.32 $\log_{10}$ in cage systems. A significant difference ($p<0.05$) was found between the mean TBC of both farming systems, with deep litter farms showing higher contamination levels. Similarly, the TEC ranged from 2.65 $\log_{10}$ to 2.80 $\log_{10}$ in deep litter farms and from 2.31 $\log_{10}$ to 2.58 $\log_{10}$ in cage systems. The mean TEC of deep litter farms was significantly higher compared to cage systems ($p<0.05$). The environmental bacterial contaminants cause contamination while the egg passes through the vent; therefore, the types of rearing and management (deep litter or cage) could play a significant role in surface microbial load and safety of eggs. The present study reported that total bacterial load (TBC) and total *Enteroacteriaceae* count (TEC) from the eggshells in deep litter system was significantly higher than in eggshells from cage system. These findings were similar to reports by previous researchers (Samiullah *et al.*, 2017, Fahim *et al.*, 2021). The probable reason for increased contamination in deep litter could be due to higher chances of contact with litter, soil and dirt of the farm in the deep litter system, while in cage system, eggs are physically segregated from the hens after laying thus reducing the probability of contamination (Hannah *et al.*, 2011).

**Occurrence of *S. aureus***

The occurrence of *S. aureus* in egg samples from deep litter farms was 30% (72/240) and from cage farms was 20% (48/240). In deep litter farms, *S. aureus* was found in 15.4% (37/240) of eggshells and 14.5% (35/240) of inner content, while in cage farms, it was present in 16.25% (39/240) of eggshells and 3.75% (9/240) of inner content. The occurrence was significantly higher in deep litter farms ($p<0.05$). The presence of *S. aureus* was influenced by farming system, feed formulation, and biosecurity, with deep litter farms using self-formulated feed and lacking biosecurity showing higher contamination. Odds of *S. aureus* contamination were 13 times higher in deep litter farms compared to cage farms, while biosecurity presence reduced contamination frequency (odds ratio: 0.295). These findings were compared with the previously reported studies where the *S. aureus* contamination on eggshells ranged from 5.5% to 14.5% (Pondit *et al.*, 2018, Parveen *et al.*, 2017, Eid *et al.*, 2015, Pyzik *et al.*, 2014, Evêncio-Luz *et al.*, 2012). The difference in the level of contamination of the eggshell surface could be attributed to the difference in geographical location of the farms, farm management practices and hygienic measures at the farm and variation in laboratory detection methods. The incidence of *S. aureus* contamination over the eggshell and inner egg content *viz*., yolk and albumen pose a potential risk of zoonotic transmission of the pathogen to the consumers (Salihu *et al.*, 2015). Previously, multiple studies have documented the occurrence of *S. aureus* on chicken meat (Abolghait *et al.*, 2020, Bernier-Lachance *et al.*, 2020, Fox *et al.*, 2017, Hanson *et al.*, 2011 and Kitai *et al.*, 2005) however, the studies exploring the incidence of *S. aureus* on eggs are relatively much lower compared to the other food products including meat.

**Occurrence of *E. coli***

In the deep litter poultry farming system, 31.25% (75/240) of egg samples were contaminated with *E. coli*, with 24.5% (59/240) and 6.6% (16/240) contamination rates for eggshells and inner content, respectively. In contrast, in cage systems, 18.75% (45/240) of egg samples were contaminated with *E. coli*, with 14.16% (34/240) and 4.5% (11/240) contamination rates for eggshells and inner content, respectively. Eggshells from deep litter systems showed significantly higher contamination compared to cage systems ($p<0.05$).

The occurrence of *E. coli* was correlated with farm characteristics, revealing that farming system and biosecurity significantly influenced *E. coli* occurrence ($p<0.001$). Higher occurrence was noted in deep litter systems without biosecurity; presence of biosecurity reduced *E. coli* occurrence, indicated by a low odds ratio (0.427, 95% C.I: 0.20-0.83). Deep litter systems harbored significantly more *E. coli* compared to cage farms ($p<0.05$), with odds of *E. coli* occurrence 1.769 times higher in deep litter compared to cage systems. These results were compared to previously documented studies where *E. coli* contamination on eggs varied across different geographical regions round the world *viz.*, 37% in Pakistan (Khan *et al.*, 2016); 50% in Southern India (Vinayananda *et al.*, 2017), 34.26% in Zambia (Kapena *et al*., 2020), 48.7% in Gauteng province of South Africa (Adesiyun *et al.*, 2020), 38.89% in Bangladesh (Hossain *et al.*, 2021); 19.8% in Western Australia (Sodagari *et al.*, 2021), 23% in Nigeria (Olopade *et al.*, 2022) and 11.7% in Punjab, India (Sharan *et al.*, 2023). Since, the major focus of surveillance in majority of the previous studies had been cloacal swabs, chicken meat and poultry litter, present study stands out to be an elaborately conducted study on eggs acknowledging the differences in contamination percentage due to difference in poultry rearing systems.

## CONCLUSION

The eggs produced under deep litter and cage farms could be a source of surface bacterial contaminants; however, the risk of such contamination increases with the exposure of eggs with infected floor surface, poultry litter material and droppings. Therefore, effective biosecurity measures and controlling hazards at the farm level will help in eliminating the risk of contaminated eggs entering the food chain.

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