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Utilization of in-feed acidifier as a control measure for *Salmonella* contamination of eggs from laying hens

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Abstract

Salmonella species are widespread in nature, and occur as pathogenic bacteria in the intestines of domestic and wild animals, including birds. Most identified Salmonella serovars are Salmonella enterica and almost all are able to cause illness in humans and animals. The environment in the laying house as well as feed contamination can serve as reservoir for Salmonella contamination of eggs. This study was designed to determine the effect of in-feed acidifier supplementation of layer's mash as a control measure for salmonella contamination of eggs. A total of 40 bovan brown layers (22 weeks old) were purchased and distributed into two treatment groups having five replicates and four birds per replicate. Birds in treatment 1 (T1) were assigned as the control group and did not have their diet supplemented with acidifier. Birds in treatment two (T2) had their diet supplemented with acidifier (Salmo-guard Pro ®) according to manufacturer's specification. Feed and water were provided ad libitum. Faecal, cloaca swab and egg samples were collected to determine Salmonella counts. Data were collected twice at two weeks interval while the experiment lasted for eight weeks. Salmonella count in collected samples was determined by pour-plate method after carrying out a serial dilution using appropriate methods with Salmonella shigella agar. Samples collected from T1 had constant and high population of Salmonella compared to T2, which had a significantly ($p<0.01$) steady reduction in the population of Salmonella in faecal, cloacal swab and egg shell over time. Supplementation of layer's diet with acidifier reduced Salmonella counts in faecal, cloacal and egg shell sampled. There was no growth of Salmonella in the yolk and albumen of eggs from both treatments. It can be concluded that Salmonella contamination of egg shell can be controlled or limited by supplementing diets of laying hens with in-feed acidifier

Keywords: Acidifier, Contamination, Eggs, laying hens, *Salmonella*

Utilisation d'un acidifiant dans l'alimentation comme mesure de contrôle de la contamination par Salmonella des œufs de poules pondeuses

Résumé

Les espèces de Salmonella sont répandues dans la nature et se présentent sous forme de bactéries pathogènes dans les intestins des animaux domestiques et sauvages, y compris les oiseaux. La plupart des sérovars de Salmonella sérovars identifiés sont Salmonella enterica et presque tous sont capables de provoquer des maladies chez les humains et les animaux. L''environnement dans le bâtiment de ponte ainsi que la contamination des aliments peuvent servir de réservoir pour la contamination des œufs par Salmonella. Cette étude a été conçue pour déterminer l''effet de la supplémentation en acidifiant de la purée de pondeuse comme mesure de contrôle de la contamination des œufs par la salmonelle. Un total de 40 pondeuses

brunes bovanes (âgées de 22 semaines) ont été achetées et réparties en deux groupes de traitement ayant cinq répétitions et quatre oiseaux par répétition. Les oiseaux du traitement 1 (T1) ont été désignés comme groupe témoin et n''ont pas eu leur alimentation complétée par un acidifiant. Les oiseaux du deuxième traitement (T2) avaient leur alimentation complétée par un acidifiant (Salmo-guard Pro ®) selon les spécifications du fabricant. La nourriture et l''eau ont été fournies à volonté. Des échantillons de matières fécales, d''écouvillons de cloaque et d''œufs ont été prélevés pour déterminer le nombre de salmonelles. Les données ont été recueillies deux fois à deux semaines d''intervalle alors que l''expérience a duré huit semaines. Le nombre de Salmonella dans les échantillons collectés a été déterminé par la méthode de la plaque de coulée après avoir effectué une dilution en série en utilisant des méthodes appropriées avec de la gélose Salmonella shigella. Les échantillons prélevés à partir de T1 présentaient une population constante et élevée de Salmonella par rapport à T2, qui présentait une réduction constante significative (p <0,01) de la population de Salmonella dans les matières fécales, les écouvillons cloacaux et la coquille d''œuf au fil du temps. La supplémentation du régime alimentaire des pondeuses avec un acidifiant a réduit le nombre de salmonelles dans les fèces, le cloaque et la coquille des œufs. Il n''y a pas eu de croissance de Salmonella dans le jaune et l''albumen des œufs des deux traitements. Il peut être conclu que la contamination par Salmonella de la coquille des œufs peut être contrôlée ou limitée en complétant les régimes alimentaires des poules pondeuses avec un acidifiant dans l''alimentation.

Mots-clés : Acidifiant, Contamination, Œufs, Poules pondeuses, *Salmonellae*

Introduction

Microbial pathogens of the genus *Salmonella* are among the leading causes of foodborne illness in the world. *Salmonella enteritidis* is one of the most prevalent foodborne pathogens. Eggs and egg-based products were frequently associated with salmonellosis outbreaks caused by *S. Enteritidis* (Braden, 2006). As concerns related to increasing human salmonellosis cases grow, the need for application of preventive ways and means either at the farm level or during the processing steps is crucial for a better control of foodborne outbreaks due to consumption of this specific food product (Anaca *et al.,* 2013). Among the different serotypes of *Salmonella enterica*, *S. Enteritidis*, and *S. Typhimurium* account for the most non-typhoidal *Salmonella* infections in both developed and developing countries (Wales and Davies, 2011). These serotypes are regarded as unrestricted, being able to cause infections in animals as well as in humans (Martelli and Davies, 2012). The environment of the laying hen house can act as reservoir for *Salmonella,* along with the feed that can be already contaminated as it arrives in the farm (Davies and Wales, 2010; Akpan and Ofongo - Abule, 2019). Due to these various sources of infection of laying hens, preventive methods already applied or available at the farm level include flock testing, sanitation and biosecurity passive immunization, use of natural antimicrobial products (bacteriophages), protein and fibre sources, competitive exclusion flora, probiotics, prebiotics, organic acids and essential oils. First approach for postharvest control of *Salmonella* in shell eggs is to maintain an adequate temperature during storage (Gantois *et al*., 2009), which is lacking in Nigeria. In addition to the discovery of *Salmonella* isolates from several livestock species, including laying birds and their environments (Abraham *et al*., 2014), there are reports of increased resistance to antimicrobial agents among isolates. *Salmonella* isolates from the shells and contents of table eggs have been

reported to exhibit resistance to antimicrobial agents (Snow *et al*., 2007). Uncontrolled therapeutic use of antimicrobial agents in the livestock industry and the use of antimicrobial agents. as additives in animal feeds to promote growth also pose food safety, public health, and therapeutic problems in animal and human diseases (Singh *et al*., 2013). Risk management and risk communication has emerged as a structured model for improving food control systems with the objectives of producing safer food, reducing the numbers of foodborne illnesses thereby facilitating domestic and international trade in food (Singh *et al.,* 2013). The need to characterize the population of *salmonella* in the cloaca, dropping and egg of layers has become relevant acknowledging that the environment of the laying hen house can act as reservoir for *Salmonella*, along with the feed which can be already contaminated (Shirota *et al.,* 2000; Akpan and Ofongo-Abule, 2019) as it arrives in the poultry farm. It's in the light of the above that this study seeks to determine the population of *Salmonella* in the cloaca, droppings and eggs of layers fed diet supplemented with acidifier.

Materials and methods

The experiment was conducted at the poultry unit, Department of Animal Sciences, Niger Delta University, Faculty of Agriculture teaching and research farm, Wilberforce Island, Bayelsa State.

Source of acidifier

The acidifier Salmo-guard pro ® used in this study was gotten from Ibadan, Oyo State. The inclusion rate as stated by the manufacturer was 500g/ton of feed in case of low contamination of feed and 400g/ton of feed for prevention purpose. The acidifier helps in control of *Salmonella* spp in raw material and finished feed, thereby avoiding chances of bacterial outbreaks in poultry flocks. The components of Salmo-guard pro are: calcium – formate, citric acid and aluminium silicate. The stated nutrient composition of the commercial layer's mash used for the study was: crude protein (16.8%), Fat (3.6%), crude fiber (4.2%), Calcium (4.2%), available phosphorus (0.5%), methionine (0.45%), lysine (0.85%), metabolizable energy (2680Kcal/kg).

Animal experiment

A total of 40 bovan brown layers (22 weeks old) were purchased from AGA Farm, Okarki, Yenagoa, Bayelsa State. The birds were randomly distributed into two treatment groups comprising five replicates with four birds per replicate. Birds in treatment 1 (T1) were assigned as the control group without having their diet supplemented with acidifier. Birds in treatment two (T2) had their diet supplemented with acidifier (Salmo-guard pro ®) which was added at the manufacturer's recommendation (500g/ton of feed). Feed and water were provided *ad libitum*. Sampling for *Salmonella* contamination of eggs was carried out for eight weeks which served as the duration of the experiment.

Sampling for Salmonella contamination

Spatula and sterile sample bottles were used to collect faecal droppings for analysis. Sterile swab sticks were used to swab inside the cloaca of birds picked at random from each replicate. One egg per replicate was also picked at random from T1 and T2 to determine *Salmonella* contamination. The data was collected in two-week interval for four times for the eight weeks experimental period. Samples were taken to the Microbiology laboratory, Department of Microbiology, Niger Delta University for *Salmonella* isolation and enumeration.

Microbial analysis

Serial dilution was carried out using one gram (1 g) of droppings, 1ml of albumen, 1m of yolk, swab of about 10cm of the egg

shell (the egg from which the albumen and yolk sample had been collected) and cloacal swab of birds on replicate basis. The serial dilution was carried out using normal saline, syringes, test-tube, test-tube rack, cutting wool, masking tape and pen. *Salmonella shigella* agar was prepared according to the manufacturer's instructions based on the calculated number of petri dishes to be used. This was placed in the autoclave for sterilization at 121ºC and 15 Pci. The agar was allowed to cool to about 45ºC, then poured into respectively labeled petri dishes (in triplicates) using pour plate method. One mill (1 mL) each from dilution factor 2- for each sample was inoculated into appropriate plastic Petri dishes labeled according to the respective treatments and replicates. The seeded agar plates were then placed in an incubator at 37ºC for 24 hours for growth and counting of *Salmonella* colonies. Number of colonies counted were log transformed, then stated as log colony forming unit (log CFU) and statistical analysis carried out using SPSS version 25.

Results and discussion

The results obtained for *Salmonella* counts (log CFU) from faecal, cloacal and egg samples collected at week 2 and 4 of the experimental period is presented in Table 1. *Salmonella* counts (log CFU) were lower in faecal, cloacal and egg samples collected from laying hens fed diet supplemented with acidifier. However, there was no growth or absence of *Salmonella* in the yolk and albumen of eggs sampled either from the control or treatment group. Samples collected from T1 had constant and high population of *Salmonella* compared to T2 which had a steady reduction ($p<0.001$) in the population of *Salmonella* over time (after four weeks of consuming acidifier supplemented diet). The population of *Salmonella* in the droppings of laying hens in T2 reduced from 3.63 log CFU (week 2) to 3.46 log CFU (week 4). This trend was also observed for *Salmonella* population recorded in the cloaca for hens under treatment 2.

Table 1: Population (log CFU) of *Salmonella* in the cloaca, droppings and egg of layers fed acidifier supplemented diet (week 2 and 4)

Sampling period	Treatments			
Week two	T1	T2	SEM	*P value*
Faecal dropping	3.83	3.63**	0.1	0.001
Cloacal swab	3.39	3.32**	0.035	0.01
Egg shell	3.84	3.51**	0.165	0.001
Egg yolk	No growth	No growth	----	
Egg albumen	No growth	No growth	----	
Week four	**T1**	**T2**	**SEM**	***P value***
Faecal dropping	3.77^{ns}	3.46^{ns}	0.155	0.951
Cloacal swab	3.42	3.25**	0.085	0.001
Egg shell	3.84	3.44**	0.195	0.001
Egg yolk	No growth	No growth	----	
Egg albumen	No growth	No growth	----	

T1: control group fed feed without acidifier;T2: treatment group fed feed supplemented with acidifier; NS: not significant ($P>0.05$); **: Significant ($P<0.01$)

Salmonella population on the egg shell was 3.84 log CFU in T1 in the first four weeks of sampling as indicated in Table 1. However, acidifier supplementation resulted in lowered ($p<0.001$) *Salmonella* population on egg shell in T2. A value of 3.51 log CFU was recorded at week 2 which was reduced to 3.44 log CFU at week 4. It was observed that the population of *Salmonella* on egg shell sampled from T1 were numerically higher than values recorded for cloaca swab sample from the same treatment. A similar observation was made for egg shell sampled under treatment 2 also. Akinola and Nwabia (2018) stated that significant higher microbial load was observed on egg shell from day 0 – 7 and up to day 14 – 21 and least on day 28. According to the authors microbial load on egg shell accumulated on the eggs due to contact with the cages and possibly the initial collection materials. An earlier EFSA report (2009), further stated that contact of eggs with contaminated surfaces such as nesting materials, dust, storage containers, handlers and the rearing environment can modify the microbial load of eggs. Although the eggs sampled in this study were collected with sterile hand gloves and placed in a sterile plastic sample bag to avoid contact with handlers or other surfaces beside the cage from which it was collected. However, the fact that its initial contact with the cage could be a contributory factor to observed results. In furtherance to this, Wang *et al*. (2011) also observed in their study that the initial microbial count on eggs shell was low but can be significantly modified by the age of boxes or nesting material where the eggs are laid. Invariably implying that eggs laid on old boxes had higher bacterial load compared to eggs laid on new boxes. These earlier reports further corroborate the results of the current study. Although the eggs were collected first thing in the morning, they were to be sampled and microbial analysis carried out on the same day. It was possible that the eggs collected had been laid late in the evening or probably at night after collection for the day sampling had ended. Furthermore, the contact of the eggs with the cage, probable faecal dropping while coming out of the cloaca and also possibly in the pen and bacteria in the environment of the pen could have contributed to the observed higher *Salmonella* population on egg shell from eggs in either treatment. Staining of eggs with faecal droppings is not a strange occurrence among laying hens. In addition, bacteria load on previously clean or new cage increases with usage thereby contaminating eggs as they come in contact with the cage during lay and before collection.

There was no growth of *Salmonella* in both yolk and albumen samples in eggs collected either from T1 or T2 as indicated in Table 1. Contamination of albumen and yolk with *Salmonella* can either be firstly due to a pre-exiting *Salmonella* infection or presences of *Salmonella* infection. Secondly and most importantly due to invasion of the inner egg contents by *Salmonella* during storage which can lead to spoilage (Akinola and Nwabia, 2018). It is also probable that the birds had no prior *Salmonella* infection which accounted for the no growth of *Salmonella* in the albumen and yolk of collected eggs. These supports the views of (Maciorowski *et al*., 2006; Wales and Davies 2011; Li *et al*., 2012).

According to earlier reports, reduction of the population of *Salmonella* contamination and spread of human salmonellosis can be achieved with preventive methods such as sanitation, disinfection and use of acidifier which can be classified as a natural antimicrobial product (Gantois *et al*, 2009; Scallan *et al*, 2011; Davies and Wales, 2012 and Anaca *et al*., 2013). Evidently, the acidifier most likely limited the growth of *Salmonella* in

the gastrointestinal tract (GIT) of the birds, thereby limiting the potential contamination of eggs with *Salmonella* as they passed through the cloaca during lay. Presence of large amount of *Salmonella* in the cloaca can be a potential source of contamination during egg lay and any effort to reduce this type of contamination could be a positive preventive measure to human salmonellosis.

The result of this study showed that there is an existence of *Salmonella* in the droppings, egg shells and cloaca of layers but not in the egg yolk and egg albumen after another four weeks of supplementing the acidifier to layer's diet. From the presented results in Table 2 below, a similar pattern as observed within the first one month of sample collection was observed. However, in this case, it is noteworthy to state that there is a further reduction ($p<0.001$) in *Salmonella* population recorded in egg samples from birds fed acidifier supplemented diet. The control group – feed only (T1) had constant and high population of *Salmonella* compared to T2 with obvious steady reduction in the population of *salmonella* over time. The population of *salmonella* in the droppings reduced from 3.63 log CFU (week 2) to 3.20 log CFU (week 8). In the cloaca, *Salmonella* population was also reduced within eight weeks (3.32 – 3.16 log CFU). The population of *Salmonella* in the egg shell was also reduced at eight weeks of treatment (3.51 – 3.21 log CFU). These findings support the views of earlier reported work by Li *et al*. (2012) and Maciorowski *et al*. (2006).

As earlier stated, no growth of *Salmonella* in the albumen and yolk of eggs sampled is of immense benefit in that, where there is no pre-existing infection in laying hens with *Salmonella*, then preventive measures targeted at feed and GIT health will be more effective to prevent human salmonellosis. This is supportive of Microbial Risk Analysis (MRA)-Risk Analysis, Risk Management and Risk Communication which has been identified as a resource intensive task requiring multi-disciplinary approach and has emerged as a structured model for improving food control system with the objectives of producing safer food, reducing the numbers of food borne illnesses as earlier reported (Singh *et al*., 2013; Anaca *et al.,* 2013; Abraham *et al.,* 2014). From the results obtained both at the sixth and eight weeks of sampling, it appears that as the amount of *Salmonella* shedding from the droppings reduce, there is a concomitant reduction in the amount of *Salmonella* present in the cloaca and on the egg shell. This observation was obvious in eggs sampled under T2. Although there was a significant ($p<0.01$) difference in *Salmonella* population on eggs sampled across both treatments, however, while the value was virtually constant in the control group, there was evident numerical reduction in *Salmonella* population in droppings, cloaca and egg shell in treatment 2 with time.

Simply put, a high count of *Salmonella* on egg shell within the first seven days after lay may not of a necessity mean the pathogen is in the egg content except a case of pre-existing infection has been established prior to determining level of contamination on egg shell cum, faecal and cloaca presence of the pathogen. However, in understanding the significance and effect of such contamination, cognizance should be taken of the fact that pathogen invasion of egg content takes place during storage (Akinola and Nwabia, 2018), of which the environment of storage plays a significant role (Aduku and Olukosi 2012). According to Aduku and Olukosi (2012), pathogen invasion during storage can be attributed to poor functioning of the eggshell under humid conditions. This further corroborates the findings of Faris *et*

Table 2: Population of *Salmonella* (log cfu) in cloaca, droppings and eggs of pullets fed acidifier supplemented diet (week six and week 8).

Week 6	T1	T2	SEM	*P value*
Droppings	3.72	3.38 **	0.27	0.001
Cloaca	3.40	3.21**	0.095	0.001
Egg shell	3.83	3.34**	0.245	0.001
Egg yolk	No growth	No growth	-	
Egg albumen	No growth	No growth	-	
Week 8	**T1**	**T2**	**SEM**	***P value***
Droppings	3.73	3.20 **	0.27	0.001
Cloaca	3.38^{ns}	3.16^{ns}	0.11	0.102
Egg shell	3.82	3.25 **	0.285	0.001
Egg yolk	No growth	No growth	-	
Egg albumen	No growth	No growth	-	

T1: control group fed feed without acidifier; T2: treatment group fedfeed supplemented with acidifier. NS: not significant (*P>0.05*); **: Significant (*P<0.01*)

al. (2011) which identified that there is usually an increase in psychrophilic bacteria, *Staphylococci*, Coliform, Molds and yeast on the shell and inner content of eggs during storage.

According to Chen *et al.* (2019), the egg cuticle is the first barrier against bacterial trans-shell penetration. It is noteworthy to also mention that freshly laid eggs have immature cuticle which is not able to resist bacterial penetration on the shell (Munoz *et al*., 2015). This lapse in the eggshell is short lived in that as the cuticles mature, they exhibited high resistance to the penetration of micro-organisms (Akinola and Nwabia, 2018). As an established fact from earlier reports (Munoz *et al*., 2015) the chemical composition of the mature cuticle determines the risk of trans-shell contamination by *Salmonella*. The mature egg cuticle which is rich in protein exhibits the highest resistance against *Salmonella* by possessing a lower permeability to penetration of *Salmonella* (Munoz *et al.,* 2015). This fact further highlights the benefit of acidifier utilization as a preventive measure to reduce or eliminate *Salmonella* contamination of eggs before the egg cuticle can act as an active barrier against *Salmonella* contamination. Furthermore, microbial growth on eggshell surface coupled with incidence of *Salmonella* penetrating the egg contents is increased in older hens (Munoz *et al*., 2015). Invariable, hen age plays a key role in microbial growth on egg shell and *Salmonella* penetration of the egg content. The hens used in this study were 22 weeks at commencement of the experiment and 30 weeks old when the study was terminated. This could also be a probable contribution to the seemingly low amount of Salmonella population recorded for eggs collected from the control group compared to values reported by Akinola and Nwabia (2018).

The acidifier used in the current study was stated to control *Salmonella* contamination of feed, and in livestock such as poultry and pigs. Its effect in reducing or limiting the amount of *Salmonella* present in faecal dropping, cloaca and egg shell of birds fed diet supplemented with Salmo-guard Pro was obvious.

Food borne *Salmonella* can contaminate poultry feed during harvesting, processing at the feed mill from dust to other ingredients and protein sources or storage. The contamination by *Salmonella* in the droppings, eggs and cloaca of layers can serve as vehicle for transmission of this pathogen which could be a great public

health risk to consumers. In the light of heightened public concern over the emergence of antibiotics resistant strains the exploitation of alternative growth to antibiotics is promoted. Acidifiers being known for their defensive effects against bacteria, fungus mold have been applied as an in-feed prophylactic measure to counter such feed pathogens in the feed industry. Thus, acidifiers in livestock nutrition are a cost-effective performance enhancing option promoting health impacts by exerting their effects through feed, GIT health, gut microbiome modification and ultimate metabolism of animals.

Conclusion

Supplementation of pullet diet with acidifier resulted in a reduction in the population of *Salmonella* in three samples which are the droppings, cloaca and egg shell over time. It also indicated no growth in the other two samples which are the egg yolk and the albumen. Prevalence of salmonellosis in human can be attributed to accumulation of *Salmonella* in the cloaca, droppings and on eggs of layers. This helps to suggest possible ways birds, eggs and droppings from poultry can be handled and treated by farmers and consumers to avoid contamination and infection from human salmonellosis.

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