ORIGINAL ARTICLE

https://doi.org/10.21708/avb.2024.18.2.12086

Effect of ammonia gas on poultry litter contaminated with *Eimeria* spp.

Efeito do gás amônia em cama aviária contaminada com Eimeria spp.

Cássio da Rosa[1], Vladimir Pinheiro do Nascimento[1], Cristiane Variani Pasqualotto[2], Laura Beatriz Rodrigues[2], Luciane Daroit[2], Fernando Pilotto[2*]

ABSTRACT: Avian coccidiosis is a common disease that affects poultry worldwide. The practices used to control this pathology in poultry are based mainly on the use of antimicrobials, which have generated increased resistance of *Eimeria* and other microorganisms. Research is therefore needed to devise new control models and strategies. The objective of our research was to evaluate the effect of different concentrations of ammonia gas on poultry litter artificially contaminated with unsporulated *Eimeria* spp. oocysts. Samples of sterilized poultry litter were placed in containers artificially contaminated with unsporulated *Eimeria* spp. oocysts. Samples were placed in plastic bags and ammonia gas was injected in concentrations of 0.2%, 0.4%, 0.8%, 1% and 1.2%. The tested dosages of ammonia gas inhibited the sporulation of oocysts in poultry litter by 100%. Our study indicates that ammonia gas is efficient in eliminating *Eimeria* spp. in poultry litter at concentrations equal to or higher than 0.2% after one hour of exposure. These results are promising because, through the use of the bedding disinfection method (shallow fermentation with ammonia injection) it will be possible to eliminate field oocysts, improving the result of coccidiosis vaccines.

KEYWORDS: avian coccidiosis; broiler chicken; biosecurity; disinfection.

RESUMO: A coccidiose aviária é uma doença avícola que afeta aves em todo o mundo. As práticas utilizadas para controlar essa patologia em aves são baseadas principalmente no uso de antimicrobianos, os quais têm gerado aumento da resistência das Eimerias e outros microrganismos. Portanto, novas pesquisas são necessárias para elaborar modelos e estratégias de controle mais eficientes. O objetivo desse estudo foi avaliar o efeito de diferentes concentrações de gás amônia em camas de frangos de corte contaminada artificialmente com oocistos de Eimeria spp. não esporulados. Amostras de camas aviárias esterilizada foram colocadas em recipientes contaminados artificialmente com oocistos de Eimeria spp. não esporulados. As amostras foram colocadas em sacos plásticos e foi injetado gás amônia nas concentrações de 0,2%, 0,4%, 0,8%, 1% e 1,2%. As dosagens testadas de gás amônia inibiram em 100% a esporulação dos oocistos, demonstrando que o gás amônia é eficiente na eliminação de Eimerias spp. em concentrações iguais ou superiores a 0,2% após uma hora de exposição. Esses resultados são promissores porque, por meio do uso do método de desinfecção da cama (fermentação na superfície com injeção de amônia), será possível eliminar oocistos de campo, melhorando o resultado das vacinas contra coccidiose.

PALAVRAS-CHAVE: coccidiose aviária; frangos de corte; biosseguridade; desinfecção.

INTRODUCTION

Avian coccidiosis, a common disease in industrial poultry farming, is caused by the protozoan *Eimeria* spp. The disease affects breeding stock, laying birds and broilers, causing losses in the zootechnical performance of birds (lower feed conversion, unevenness and low weight gain) (Assis; Luns; Cury, 2013). Global losses caused by this disease are estimated to reach approximately U$ 10.4 billion per year (Blake *et al.*, 2020).

Eimeria spp. are intracellular parasites with inherent characteristics, such as the presence of an apical complex formed by an apical ring, conoids, micronemes, rhoptries and micropores. Conoids are responsible for penetrating the cell, while the other organelles secrete enzymatic substances (Kawazoe, 2009). Upon infection, *Eimeria* spp. damage the intestinal mucosa, thereby reducing the absorption of food nutrients (Gabriel *et al.*, 2006; Pinheiro *et al.*,

[1]Universidade Federal do Rio Grande do Sul, Porto Alegre/RS, Brasil
[2]Universidade de Passo Fundo, Passo Fundo/RS, Brasil
*Corresponding author: fernandopilotto@upf.br
Received: 07/24/2023. Accepted: 11/06/2023

2014). A variety of *Eimeria* species can affect avian health. In Brazil, the most prevalent species in broilers are *E. acervulina*, *E. maxima* and *E. tenella*. Other parasite species that infect commercial egg-laying birds are *E. necatrix*, *E. brunetti*, *E. praecox* and *E. mitis* (Kawazoe, 2009). The subclinical form of coccidiosis is observed in most cases of the disease. However, in acute cases, birds exhibit more relevant symptoms such as mucoid or bloody diarrhea, dehydration, skin depigmentation, prostration, weight loss, susceptibility to secondary infections and death (Pinheiro *et al.*, 2014). *Eimeria* spp. oocysts are quite resistant to the environment, multiplying rapidly under favorable conditions of temperature, humidity and oxygen (Lovato, 2018). In addition, the increasing application of preventive drugs in poultry feed (anticoccidials) has been raising growing concern regarding the development of antimicrobial resistance and the presence of drug residues in poultry meat products (Lovato, 2018; Ojimelukwe *et al.*, 2018).

In this context, alternative methods are needed in place of anticoccidials to control coccidiosis. Ammonia (NH_3) gas has been proven effective in destroying *Eimeria* spp. oocysts in high concentrations (Horton-Smith; Taylor; Turtle, 1940; Chroustová; Pinka, 1987; Fayer; Graczyk; Trout, 1996; Lorenzoni, 2020). Ammonia, a chemical compound commonly used in industries, is widely employed in refrigeration systems due to its low cost. Some industrial segments also use ammonia in the form of quaternary ammonium as a disinfectant (Von-Wirén; Merrick, 2004). In poultry production, ammonia originates from the microbial fermentation of uric acid present in poultry litter. Uric acid undergoes the action of the enzyme uricase produced by bacteria, which converts it into ammonia and carbon dioxide (Kim; Patterson, 2003). The mechanism of action of ammonia is still not clearly understood. It is known that NH_3 can easily penetrate the cell wall in animal cells. Inside the cell, NH_3 is believed to act by increasing intracellular pH through direct influx, binding to hydrogen ions and pushing potassium out of the cell, thus leading to the destabilization of cell homeostasis (Warren, 1962; Lovato, 2018). The new method of disinfecting poultry litter by covering its surface with a plastic sheet and applying a controlled injection of ammonia gas at a concentration of 1% into the litter has been shown to be effective in eliminating *Salmonella* Heidelberg, Typhimurium and Enteritidis in experiments carried out *in vitro* and in the field (Mendonça *et al.*, 2021). However, as yet there are no studies that can support the effectiveness of ammonia gas against *Eimeria* spp. and other microorganisms (Luther, 2015; Decrey *et al.*, 2016; Gehring *et al.*, 2020). The purpose of our research was to evaluate the effect of different concentrations (0.2%, 0.4%, 0.8%, 1% and 1.2%) of ammonia gas injected in a controlled manner into poultry litter artificially contaminated with *Eimeria* spp.

MATERIAL AND METHODS

The experiment was carried out at the Center for Diagnostics and Research in Animal Health (CDSA) at the University of Passo Fundo (UPF) and was approved by the university's Animal Research Ethics Committee, under Protocol No. 015/2020. Sixteen 10-day-old Cobb 500 broiler chickens vaccinated against Marek disease, Gumboro disease, infectious bronchitis and fowl pox were housed in suitable conditions on a floor covered with wood shavings at the Center for Agricultural Research and Extension (CEPAGRO) of UPF. The broilers were fed maize and soybean meal without anticoccidials and had free access to drinking water. At the age of 12 days, the broilers were inoculated by means of an oral vaccine containing sporulated oocysts (Costa; Paiva, 2009). The sporulated oocysts of *Eimeria* spp. came from a live attenuated Bio-Coccivet R® vaccine containing the following strains: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. praecox*, *E. tenella* and *E. mitis*, which were isolated from field strains in Brazil. The birds were given an experimental inoculation of 0.6 mL of vaccine containing $20x10^5$ sporulated oocysts through an esophageal cannula coupled to a 1mL syringe. One hundred and forty-four hours after inoculation, bird feces were collected and the oocysts were quantified as sporulated and unsporulated, according to the modified technique developed by Gordon and Whitlock (1939). We counted 125,000 oocysts/gram of feces, all of them in the unsporulated phase. All the procedures were performed and materials were collected and kept at a temperature of 4°C to 7°C. After the quantification and evaluation of unsporulated oocysts, the oocyst inoculum was prepared by diluting the total volume of feces (600 mL) in a 2.5% potassium dichromate solution. Previously sterilized 0.5-kg samples of poultry litter from broiler production were placed in sterile plastic containers. The samples were inoculated with 25 mL of inoculum containing 125,000 oocysts/mL of *Eimeria* spp. and thoroughly homogenized. The depth of litter in the plastic containers was approximately 20 cm to simulate the condition observed at the poultry farm. Subsequently, the samples were placed in a plastic bag and ammonia gas in concentrations of 0.2%, 0.4%, 0.8%, 1% and 1.2% was injected into it. The amount of ammonia gas injected into the samples was measured based on the reduction in weight of the gas cylinder, which was weighed on a digital precision scale. After one hour, the plastic bags were opened, and the samples kept at a temperature of 25°C to stimulate oocyst sporulation. All the treatments, including positive (poultry litter + inoculum) and negative (poultry litter) controls, were tested in quadruplicate. After 72 hours in a laminar flow hood, 50 grams of poultry litter were collected from each sample and subjected to the modified technique of Gordon and Whitlock (1939) for oocyst isolation and quantification. The morphology of the *Eimeria* spp. oocysts was examined under an optical microscope. The data were evaluated by Dunn's post-hoc test and the Kruskal-Wallis test ($P > 0.05$), and the means were compared by the Mann-Whitney test ($P > 0.05$).

RESULTS

Ammonia gas concentrations of 0.2%, 0.4%, 0.8%, 1% to 1.2% inhibited the sporulation of *Eimeria* spp. oocysts by 100% in reused poultry litter (Table 1). As expected, oocyst sporulation occurred in the positive control but not in the negative control. Lower counts of unsporulated oocysts were observed in response to higher concentrations of ammonia gas (P <0.001). We attribute this lower count to the rapid elimination of oocysts by ammonia gas, which may have caused their deterioration. The Gordon and Whitlock (1939) method requires the oocyst wall to be intact to enable it to float when placed in a hypersaturated glucose solution and to be identified.

DISCUSSION

The hypothesis that ammonia gas in different concentrations can inhibit the sporulation of *Eimeria* spp. oocysts in contaminated poultry litter was confirmed here and is in agreement with reports published in the literature.

Horton-Smith, Taylor and Turtle (1940) applied a concentration of 25 ppm of ammonia gas and eliminated 100% of oocysts in an oocyst culture. Chroustova and Pinka (1987) reported that the application of a 0.5% concentration of ammonium hydroxide and ammonia gas for 24 hours inhibited oocyst sporulation. These authors conclude that the action of ammonia lies in its high level of toxicity, regardless of the pH of the solution.

Fayer, Graczyk and Trout (1996) demonstrated the efficiency of ammonia gas and other low molecular weight compounds against *Cryptosporidium parvum* oocysts, leading to loss of viability and infectivity with a 5% concentration in 18 hours of treatment. Lorenzoni (2020) reported the sensitivity of *Eimeria* spp. oocysts in the environment, especially when they are outside the host for a period of two or more weeks. The author also defends the need to use an effective disinfection protocol for reused poultry litter in order to reduce the oocyst load in the environment, as well as a vaccination program. This strategy can generate positive results, providing new alternatives for the control of avian coccidiosis.

According to Dai Prá *et al.* (2009) and Muniz *et al.* (2014), the use of disinfection methods for reused poultry litter (the addition of lime, windrowing, and covering the surface with plastic sheeting) act on the pH level, ammonia volatilization and nitrogen retention of the litter, thereby keeping its moisture levels low and hindering the sporulation of *Eimeria* spp. oocysts. However, these factors must interact effectively to increase the production of ammonia, which acts against microorganisms (Oliveira; Ferreira; Cancherini, 2004; Ferreira; Oliveira; Traldi, 2004; Soliman; Sallam; Abouelhassan, 2018).

Our results are substantiated by Garcés-Gudiño, Merino-Guzmán and Cevallos-Gordó (2018), who reported that new litter contained larger numbers of oocysts than reused broiler litter. The greater production of ammonia in reused litter may be responsible for this reduction in the number of oocysts. Our study also reinforces the results obtained by Chapman and Johnson (1992) and Assis, Luns and Cury (2013), who reported a significant reduction in the number of *Eimeria acervulina* oocysts when poultry litter was subjected to fermentation treatment with windrowing and plastic sheet covering for 10 days.

Another important factor involves the findings reported by Kawazoe (2009), which indicate the resistance of the oocyst wall of *Eimeria* spp. to proteolysis and its impermeability to water-soluble substances, including to the different disinfectants commonly used in the poultry chain. However and Kawazoe (2009) described the effectiveness of some low molecular weight chemical compounds in eliminating oocysts, including ammonia gas, methyl bromide and various phenolic compounds. Kim and Patterson (2003) reported that the addition of limestone or hydrated lime on poultry litter contributes to raise its pH level, improving the action of the enzyme uricase and thus increasing the production of ammonia.

Reece, Lott and Deaton (1980) and Payne *et al.* (2007) observed that ammonia levels in litter are low when the pH is below 7. This factor contributes to greater ammonium production, which has low antimicrobial activity. Mendonça *et al.* (2021) employed the method of plastic sheet covering on the surface combined with the controlled injection of ammonia gas and found that

Table 1. Disinfectant effect of different concentrations of ammonia gas in poultry litter contaminated with *Eimeria* spp.

	Number of Sporulated Oocysts/gram of feces ($\underline{x} \pm s$)	Number of Unsporulated Oocysts/gram of feces ($\underline{x} \pm s$)	*P*-value
Positive Control	105,617.00 ± 656.39 ^{aA}	0.00 ± 0.00 ^{aB}	< 0.001
NH_3 concentration - 0.2%	0.00 ± 0.00 ^{bA}	72,202.00 ± 107.09 ^{bB}	< 0.001
NH_3 concentration - 0.4%	0.00 ± 0.00 ^{bA}	66,297.25 ± 112.40 ^{cB}	< 0.001
NH_3 concentration - 0.8%	0.00 ± 0.00 ^{bA}	46,004.00 ± 1,536.76 ^{dB}	< 0.001
NH_3 concentration -1.0%	0.00 ± 0.00 ^{bA}	33,995.00 ± 627.37 ^{eB}	< 0.001
NH_3 concentration - 1.2%	0.00 ± 0.00 ^{bA}	25,214.25 ± 131.55 ^{fB}	< 0.001
P-value	< 0.001	< 0.001	----------

^{a,f} Means in the columns differ by Dunn's post-hoc test from the Kruskal-Wallis test (p> 0.05).
^{A,B} Means in the rows differ by the Mann-Whitney test (p> 0.05).

a 1% concentration suffices to eliminate *Salmonella* Heidelberg, *S.* Typhimurium and *S.* Enteritidis in contaminated poultry litter. The controlled injection of ammonia gas into poultry litter covered with plastic sheeting is a method that can enhance the disinfection of reused litter, deactivating the physicochemical parameters that interact and interfere in the natural production process of ammonia gas by microbial fermentation (Mendonça *et al*., 2021). In addition, the method of plastic sheeting on poultry litter combined with the injection of ammonia into the litter reduces the disinfection period to two days compared to the 10 days required by traditional methods (lime addition, plastic sheeting on litter surface and windrowing), increasing the profitability and economic viability of poultry production (Rosa, 2014; Mendonça *et al*., 2021). Our findings demonstrate that concentrations equal to or higher than 0.2% ammonia ensure the elimination and inhibition of sporulation of *Eimeria* spp. oocysts in 20-cm-thick layers of reused poultry litter within a period of 48 hours.

The results of this study contribute to improve the method described herein to control the proliferation of *Eimeria* spp. in reused poultry litter. Our findings are also expected contribute to reduce the use of anticoccidials, ensuring consumers have access to safer food free of chemical residues and antimicrobial-resistant parasites.

CONCLUSION

Ammonia gas in concentrations equal to or greater than 0.2% eliminates coccidiosis oocysts in poultry litter after an hour of exposure. The data obtained in this work are promising, enabling a new way of controlling avian coccidiosis.

ACKNOWLEDGEMENTS

The University of Passo Fundo, University Federal of Rio Grande do Sul and Aveclean for the financial support in carrying out this research.

REFERENCES

ASSIS, R. C. L.; LUNS, F. L.; CURY, M. C. Disinfection with quaternary ammonia associated with fermentation enhances do not control coccidiosis in poultry litter. **Ciência Rural**, v. 43, p. 1459-1463, 2013. Disponível em: https:/doi.org/10.1590/S0103-84782013005000103.

BLAKE, P. D. *et al*. Re-calculating the cost of coccidiosis in chickens. **Veterinary Research**, v. 51, p. 1-14, 2020. Disponível em: https:/doi.org/10.1186/s13567-020-0837-2.

CHAPMAN, H. D.; JOHNSON, Z. B. Oocysts of *Eimeria* in the litter of broilers reared to eight weeks of age before and after withdrawal of lasalocid or salinomycin. **Poultry Science**, v. 71, p. 1342-1347, 1992. Disponível em: https:/doi.org/10.3382/ps.0711342.

CHROUSTOVÁ, E.; PINKA, K. The Efficiency of Disinfectants on the Oocysts of *Eimeria tenella*. **Veterinary Research**, v. 5 p. 141-149, 1987.

COSTA, C. A. F.; PAIVA, D.P. **Cultivo in vivo, in vitro e diagnóstico específico de *Eimeria* spp. de *Gallus gallus***. Brasília: Embrapa Informação Tecnológica, Brasil. 2009.

DAI PRÁ, M. A. *et al*. Uso de cal virgem para o controle de *Salmonella* spp. e *Clostridium* spp. em camas de aviário. **Ciência Rural**, v. 39, p. 1189-1194, 2009. Disponível em: https:/doi.org/10.1590/S0103-84782009005000028.

DECREY, L. S.; KOHN, T. Ammonia as an *in situ* sanitizer: influence of virus genome type on inactivation. **Applied and Environmental Microbiology**, v. 82, p. 4909–4920, 2016. Disponível em: https:/doi.org/10.1128/AEM.01106-16.

FAYER, R. T. K.; GRACZYK, M. R.; TROUT, J. M. Gaseous disinfection of *Cryptosporidium parvumoocysts*. **Applied and Environmental Microbiology**, v. 62, p. 3908–39, 1996. Disponível em: https:/doi.org/10.1128/AEM.62.10.3908-3909.

FERREIRA, H. A.; OLIVEIRA, M. C.; TRALDI, A. B. Efeito de condicionadores químicos na cama de frango sobre o desempenho de frangos de corte. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 56, p. 542-546, 2004. Disponível em: https:/doi.org/10.1590/S0102-09352004000400017.

GABRIEL, L. *et al*. Effects of whole wheat feeding on the development of coccidial infection in broiler chickens until market-age. **Animal Feed Science and Technology**, v. 129, p. 279-303, 2006. Disponível em: https:/doi.org/10.1016/j.anifeedsci.2006.01.004.

GARCÉS-GUDIÑO, J.; MERINO-GUZMÁN, R.; CEVALLOS-GORDÓN, A. L. Litter reuse reduces *Eimeria* spp. oocyst counts and improves the performance in broiler chickens reared in a tropical zone in Ecuador. **European Poultry Science**, v. 82, 2018. Disponível em: https:/doi.org/10.1399/eps.2018.220.

GEHRING, V. S. *et al*. *Alphitobius diaperinus* control and physicochemical study of poultry litters treated with quicklime and shallow fermentation. **Poultry Science**, v. 99, p. 2120–2124, 2020. Disponível em: https:/doi.org/10.1016/j.psj.2019.11.039.

GORDON, H. M. C. L.; WHITLOCK, H. V. A new technique for counting nematode eggs in sheep faeces. **Journal of Scientific and Industrial Research**., v. 12, p. 50-53, 1939.

HORTON-SMITH, C.; TAYLOR, E. L.; TURTLE, E. E. Ammonia Fumigation for Coccidia Disinfection. **Veterinary Record**, v. 52, p. 829-832, 1940.

KAWAZOE, U. Coccidiose. In: BERCHIERI JUNIOR A.; MACARI, M. **Doenças das Aves**. 2. Ed. Fundação APINCO de Ciências e Tecnologia Avícola (APINCO). Campinas- SP, cap. 7, p. 391-423, 2009.

KIM, W. K.; PATTERSON, P. H. Effect of minerals on activity of microbial uricase to reduce ammonia volatilization in poultry manure. **Poultry Science**, v. 82, p. 223-231, 2003. Disponível em: https:/doi.org/10.1093/ps/82.2.223.

LORENZONI, G. **Avian Coccidiosis**. PennState Extension, 2020. Disponível em: https:/extension.psu.edu/avian-coccidiosis.

LOVATO, M. Coccidiose. **Doenças das Aves**. São Paulo: Kindle Direct Publishing. 2018, p. 141-149.

LUTHER, A. K. **Ammonia toxicity in bacteria and its implications for treatment of and resource recovery from highly nitrogenous organic wastes**. Doctoral Thesis. New Jersey State University. 2015. Disponível em: https:/doi.org/10.7282/T3668G53.

MENDONÇA, B. S. *et al*. Use of ammonia gas for *Salmonella* control in poultry litters. **Poultry Science**, v. 100, p. 314-318, 2021. Disponível em: https:/doi.org/10.1016/j.psj.2020.10.008.

MUNIZ, E. *et al*. Presence of *Salmonella* spp. in reused broiler litter. **Revista Colombiana de Ciencias Pecuarias**, v. 27, p. 12-17. 2014.

OJIMELUKWE, A. E. *et al*. Populations of *Eimeria tenella* express resistance to commonly used anticoccidial drugs in southern Nigeria. **International Journal of Veterinary Science and Medicine**, v. 6, p. 192-200, 2018. Disponível em: https:/doi.org/10.1016/j.ijvsm.2018.06.003.

OLIVEIRA, M. C.; FERREIRA, H. A.; CANCHERINI, L. C. Efeito de condicionadores químicos sobre a qualidade da cama de frango. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 56, p. 536-541, 2004. Disponível em: https:/doi.org/10.1590/S0102-09352004000400016.

PAYNE, J. B. *et al*. Modling the growth and dead kineties of *Salmonella* in poultry litter as a function of pH and water activity. **Poultry Science**, v. 86, p. 191-201, 2007. Disponível em: https:/doi.org/10.1093/ps/86.1.191.

PINHEIRO, B. *et al*. Coccidiose em Frangos de Produção. **Revista Científica de Medicina Veterinária**, Ano XII, n. 22, 2014.

REECE, F. N.; LOTT, B. D.; DEATON, J. W. Ammonia in the atmosphere during brooding affects performance of broiler chickens. **Poultry Science**, v. 59, p. 486-488, 1980. Disponível em: https:/doi.org/10.3382/ps.0590486.

ROSA, P. S. Cama para frangos de corte. **Produção de frangos de corte**. 2 ed. FACTA: Campinas. 2014; cap. 9, p. 153-180.

SOLIMAN, E. S.; SALLAM, N. H.; ABOUELHASSAN, E. M. Effectiveness of poultry litter amendments on bacterial survival and *Eimeria* oocyst sporulation. **Veterinary World**, v. 11, p. 1064-1073, 2018. Disponível em: https:/doi.org/10.14202%2Fvetworld.2018.1064-1073.

VON-WIRÉN, N.; MERRICK, M. Regulation and function of ammonium carriers in bacteria, fungi, and plants. Molecular: Mechanisms Controlling Transmembrane Transport. In: **Topics in Current Genetics.** Springer, Berlin, Heidelberg., v. 95, p. 120, 2004.

WARREN, K.S. Ammonia toxicity and pH. **Nature**, v. 195, p. 47-49, 1962. Disponível em: https:/doi.org/10.1038/195047a0.