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Original Article

Effect of Ammonia Gas in Poultry Litter Contaminated with Salmonella Heidelberg

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ABSTRACT

Salmonella Heidelberg is an emerging pathogen in Brazilian poultry production. The traditional methods (quicklime, windrowing and tarpaulin-on-surface) used for disinfecting reused poultry litter between flocks does not guarantee its elimination, thus allowing the transmission of this agent from one flock to another. The new tarpaulin-on-surface method with controlled injection of ammonia gas has proven to be effective in its control, however, it is still unknown what dose of ammonia gas is needed to eliminate *Salmonella* Heidelberg in reused poultry litter. The objective of this study was to evaluate the effect of ammonia gas at different concentrations in sterile poultry litter artificially contaminated with *Salmonella* Heidelberg. Then, ammonia gas was injected in concentrations of 0.25%, 0.5%, and 1%, and 48 hours later, a sample was collected from each repetition in an entirely randomized design, and bacterial isolation was performed. All treatments, including positive and negative controls, were tested in quadruplicate and the parameters temperature, humidity, pH and water activity were evaluated. In the 0.5% and 1% treated samples the pathogen was not isolated, while in the 0.25% concentration one of the four samples tested was positive. The study reveals that ammonia gas is efficient in killing *Salmonella* Heidelberg in poultry litter at concentrations of 0.5 % or more within a 48-hour period and that the litter treated with ammonia gas increases its pH and water activity.

INTRODUCTION

Poultry litter reuse is a practice commonly adopted throughout Brazil due to the scarcity of material, high replacement cost and environmental contamination, but it can lead to serious chemical and microbiological contamination problems of water and soil resources, putting at risk the quality of life of the population around the production units. The techniques commonly used for the disinfection of reused poultry litter exhibit little efficiency in controlling pathogens such as Salmonella and mainly in preventing reinfection between lots, requiring further studies (Orrico *et al.*, 2015; Andrade, 2017).

Salmonella is a microorganism with natural habitat in the gastrointestinal tract of birds, which may involve greater environmental dissemination and possible reinfection of birds (Vaz *et al.*, 2017; Mendonça *et al.*, 2021).

Due to its high prevalence, resistance to antimicrobials and difficult control, *Salmonella* Heidelberg has been isolated and reported in Brazil, from poultry and products derived from chicken meat since 1962. *Salmonella* stands out as one of the main serovars causing infections in humans worldwide, occupying the fourth position among the most isolated ones (Vieira *et al.*, 2009; Vieira *et al.*, 2015).

In recent years, a higher incidence of *Salmonella enterica* subspecies *enterica* serovar *Heidelberg* (*S.* Heidelberg) has been observed in poultry houses and processing plants by *Salmonella* control and monitoring programs (Ferrari *et al.*, 2019). According to data from the World Health Organization (WHO, 2017b) in the Global Salm-Surv (GSS) program, *S.* Heidelberg was among the 15 most commonly isolated serovars in animal samples, environment, and animal feed in Brazil until 2012. It is one of the most detected serovars in humans and most prevalent in food intended for human consumption. In addition, *S.* Heidelberg was also among the most serotyped serovars between the years 2013 to 2014 and 2016 to 2018, according to the Rapid Alert System for Food and Feed (WHO, 2017a). This growing importance has led to studies due to the strong resistance to antimicrobial drugs, such as ceftiofur and ceftriaxone, limiting human treatment options of salmonellosis (PHACASPC, 2007; Robinsom, 2013; Shah *et al.*, 2017).

Based on many described difficulties facing *S.* Heidelberg in poultry litter, specific studies targeting new methodologies and tools to control this pathogen are essential. In this context, further research and improvement of knowledge related to the action of ammonia gas in poultry litter contaminated with different pathogens are sought.

Ammonia is a chemical compound commonly used in different industries, with large applicability in the refrigeration sector. In addition, it is used in the quaternary ammonium form as a disinfectant in some industrial segments (Von, 2004).

In relation to poultry production, ammonia originates from the accumulation of bird excreta, which has high uric acid content. After undergoing the action of the enzyme uricase, bird excreta is converted to allantoic acid and then converted to ureidoglycolate. After this step, along with glyoxylate and urea, it is hydrolyzed to NH_3 and carbon dioxide (Kim & Patterson, 2003). The broiler production systems favor the production of NH_3, given the contributing factors: high densities of broilers in poultry farms, the type of litter used and the small interval between flocks. Another contributing factor is the adopted diets, with approximately 55% of the nitrogen present in the feed being able to be excreted in the poultry litter (Egute *et al.*, 2010; Silva, 2011).

The mechanism of action of intracellular NH_3 is not yet clearly understood and elucidated. It is known that NH_3 can reach and cross the bacterial cell wall in animal cells (Warren, 1962). Inside the bacterial cell, it is believed that NH_3 acts by increasing the intracellular pH through direct influx, binding to hydrogen ions and displacing potassium to outside the cell, causing destabilization of cellular homeostasis. Thus, the compound has been analyzed against different microorganisms. Nevertheless, there is still a lack of studies that supports the efficiency of the compound, in the form of gas or fumigation, against *Salmonella* and other pathogens (Luther, 2015; Decrey *et al.*, 2016; Gehring *et al.*, 2020). Thus, the objective of our study was to evaluate the action of ammonia gas injected in a controlled manner at concentrations of 0.25%, 0.5% and 1% in poultry litter artificially contaminated with *S.* Heidelberg.

MATERIALS AND METHODS

The experiment was carried out at the Animal Health Diagnosis and Research Center (CDSA) of the University of Passo Fundo (UPF) and approved by the Animal Ethics Committee of the University of Passo Fundo (registration number 015/2020).

The strain of *S.* Heidelberg was obtained from an isolated field from the litter of a poultry company. The poultry litter samples used in the experiment had been recycled during 11 flocks and were sterilized in an autoclave to avoid interfering with the normal litter microbiota in the ammonia effect assessment, submitting *Salmonella* which are fastidious micro-organisms. The *S.* Heidelberg strain was reactivated in Brain Heart Infusion (BHI) broth and incubated at 37 ±1°C for 24 hours. Five drops of 10µL of the sample were inoculated into Petri dishes containing Plate Count Agar (PCA) and then incubated at 37°C/24h. The score was multiplied by 20 and by the dilution factor, obtaining the result of 2.58 x 10^9 CFU/ml of *S.* Heidelberg. The biochemical identification of *Salmonella* was also carried out (ISO 6579, 2002). The biochemicals used were TSI (Triple Sugar Iron Agar), LIA (Lysine Iron Agar), SIM and Urea. All culture media used in the experiment were Merck and Oxoid brands.

Previously sterilized litter samples containing 4 kg portions were divided into the treatments and placed in sterile plastic bags and contaminated with 1 mL of bacterial culture containing 2.58 x 10^9 CFU/ml of *S.* Heidelberg. Samples were homogenized in several directions. The litter thickness in the plastic bags was approximately 20 cm to simulate a condition similar to the one observed in the poultry farm. Ammonia gas was injected at concentrations of 0.25%, 0.5%, and 1%. For application, a cylinder containing 01 kg

of ammonia was placed on top of an electronic scale. In this cylinder, a valve is placed, which when opened starts to inject through a hose, ammonia into the plastic bags that contained the litter samples. The amount injected is calculated by the difference in weight seen on the scale's display before the valve is opened and until its closing, making it possible to place the desired volume of ammonia.

The amount of ammonia gas injected into the samples was measured and verified by weight reduction of the gas cylinder placed on a precision digital scale. All treatments, including positive (litter + inoculum) and negative (litter) controls, were tested in quadruplicate (4 samples per treatment), totalizing 20 samples. After 48 hours, the plastic bags were opened in a laminar flow hood and 25 g of poultry litter was collected from each sample. Collected samples were homogenized in 225 mL of 1% soy peptone broth with the aid of a mechanical stirrer and maintained in a bacteriological incubator at 37ºC ±2°C for 24 hours. Then, 0.1- and 1.0-mL aliquots were removed and transferred to 9.9 mL of tetrathionate broth and 9 mL of Rappapport-Vassialidis broth respectively. The incubated broths were plated onto at respectively 37ºC and 42ºC for 24 hours. The samples were removed and streaked on xylose-lysine-tergitol-4 agar, MacConkey agar and Brilliant-green Phenol-red Lactose Sucrose (BPLS) agar for isolation of typical *Salmonella* isolates. Biochemical tests were performed to confirm typical colonies of *Salmonella* spp. The methodology used for diagnosis was the conventional method (ISO 6579:2017) because this is a more specific test and sensitive in relation to bacterial counting methods.

To measure the physicochemical parameters of the poultry litter, the thermometer was used to measure the temperature. AKSO digital dispositive (±1°C precision). Humidity was evaluated by weight difference between samples after drying in 55ºC/48h and pH was measured diluting 10g of samples in 50mL of Calcium Chloride, homogenizing for 30 min and using digital pHmeter. Water activity was measured using Testo 650 (ITCER-20).

For statistical analysis of the data, the Dunn test - Kruskal-Wallis post-hoc test ($p<0.05$) was used. This test compares the different observations and detects the different results between the treatment group. In the statistical analysis of the variables AW, pH, Humidity and temperature, the Analysis of Variance (ANOVA) was used, which is based on the decomposition of the total variation of the response variable into plots that can be associated with the treatments (variance between) and the associated experimental error (inside variance). The model used was: $Yij = \mu + Ti + Eij$, where Yij = observation of the i-th treatment in the j-th experimental unit; μ = overall mean; Ti = treatment effect and Eij = associated error. Tukey's test was used to compare the means of treatment with a significance level of 5% ($\alpha = 0.05$) and significance established at $p \leq 0.05$. Statistical analysis was performed using SPSS 23 software.

RESULTS AND DISCUSSION

The results from this study are shown in Table 1. Ammonia gas at concentrations of 0.5 and 1% exhibited 100% effectiveness in eliminating *S.* Heidelberg. Although the 0.25% concentration had no statistical difference from the 0.5 and 1% treatments, one of the four samples tested at 0.25% concentration showed a positive result.

Table 1 – Disinfectant effect of ammonia gas at different concentrations in poultry litter contaminated with *Salmonella* Heidelberg.

	Positive Samples	*p*-value
Positive control	4 (100%) a	
Negative control	0 (0%) b	
NH3 concentration - 0.25%	1 (25%) b	0.004
NH3 concentration – 0.5%	0 (0%) b	
NH3 concentration – 1.0%	0 (0%) b	

[a,b] Values followed by different superscripts differ significantly ($p>0.05$) by Dunn's test - Kruskal-Wallis post-hoc.

The hypothesis that different concentrations of ammonia gas could be efficient in eliminating *S.* Heidelberg in contaminated poultry litter has been confirmed. The results obtained in this study corroborate with a study by Mendonça *et al.* (2021) that reported the effectiveness of 1% ammonia gas to inactivate different *Salmonella* serovars, including *S.* Heidelberg. In this same study under field conditions, the concentration of approximately 0.24% was effective in eliminating *S.* Heidelberg in poultry litter contaminated by these bacteria. However, the litter thickness of the tested poultry farm was 10 cm, whereas a larger thickness of 20 cm was used in the present research. Due to ammonia gas being volatile, we consider that the greater the thickness of the litter, the greater the dose of injected ammonia gas should be to disinfect the deeper litter layers (Chen *et al.*, 2015; Mendonça *et al.*, 2021). On the other hand, Lopes *et al.* (2015) observed that the bacterial concentration is higher in the most superficial layers of the litter under field conditions.

Voss-Rech *et al.* (2017) found that covering the litter on the surface with plastic, without windrowing, for 10 days to stimulate bacteriological fermentation, generated 0.28% ammonia concentration and was not efficient in eliminating *S.* Heildeberg in artificially contaminated poultry litter. The Kjeldhal method was used in this study to detect the quantity of ammonia present in the poultry litter. And also, this method is able to detect the presence of ammonium nitrogen in the litter. Warren (1962) demonstrated that ammonia (NH_3) passes easily through cell membranes, while ammonium (NH_4^+) has a low penetration and reduced action as a disinfectant.

Koziel *et al.* (2017) and Himathongkham & Riemann (1999) observed that the minimum inhibitory concentration of ammonia to eliminate *Salmonella* Typhimurium, *Staphylococcus aureus* and other resistant microorganisms in animal carcasses digested in a period of 24 hours was respectively of 0.148% and 0.734%. These findings demonstrated that the bacteria present different degrees of sensitivity to the disinfectant action of ammonia.

The disinfectant action of ammonia is still not clearly understood. It is known that NH_3 entry into the cell generates a destabilization of cellular homeostasis due to the increase in intracellular pH (Warren, 1962). Therefore, there are several physical-chemical factors that can interfere with the efficiency of methods for disinfecting reused litter (Gehring *et al.*, 2020). Factors such as humidity, temperature, microbial infection pressure and pH can directly interfere with fermentation and natural production of ammonia gas (Trabulsi & Alterthum, 2015; Turnbull & Snoevenbos, 1973).

Egute *et al.* (2010) found less ammonia production in litters with high humidity due to the increased formation of ammonium and reduced growth of microbial and enzyme activity. Ottoson *et al.* (2008) and Traldi *et al.* (2007) reported that the reused poultry litter has higher pH values and volatilized ammonia when compared to the poultry litter of a first flock. This can be explained by the accumulation of uric acid in the litter that occurs with increased number of flocks reared out on the litter. Besides, the addition of limestone or hydrated lime on the litter contributes to pH elevation, improving the action of the uricase enzyme and consequently increasing ammonia production (Kim & Patterson, 2003). Reece *et al.* (1980) observed that ammonia levels are low in the litter when the pH is below 7. This factor leads to greater ammonium production, which has low antimicrobial activity (Payne *et al.*, 2007).

In this way, the controlled injection of ammonia gas within the surface of litter covered by plastic in broiler houses during downtime between flocks is a method that can improve the disinfection of reused litters, isolating factors such as the physical-chemical parameters that interact and interfere in the ammonia gas production process by microbial fermentation (Mendonça *et al.*, 2021). In addition, the tarpaulin-on-surface with controlled ammonia injection might reduce the disinfection period from 10 to two days when compared to traditional methods (lime addition, tarpaulin-on-surface and windrowing). This method can generate greater profitability and economic viability in poultry production (Rosa, 2014; Chen *et al.*, 2015; Mendonça *et al.,* 2021).

Our results showed that the dose of 0.5% ammonia guarantees the elimination of *S.* Heidelberg in reused litter with a litter thickness of 20 cm during a period of 48 hours. Nevertheless, the dose can be adjusted depending on the litter thickness and the application time. Koziel *et al.* (2017) demonstrated that the longer the exposure time of ammonia to *Salmonella* Typhimurium, the greater its disinfectant effect. Our findings clarify why the method tarpaulin-on-surface does not guarantee the litter disinfection. This method generates a maximum of 0.1% ammonia gas concentration through the bacterial fermentation process, while the amount required to guarantee the elimination of *S.* Heidelberg must be greater than 0.5%. As for the use of ammonia in the treatment of poultry litter, it is a very safe method regarding the environment. Much of the injected ammonia is incorporated into the surface as ammonia and improves its quality as a soil fertilizer. Also, Muniz *et al*. (2022), treating poultry litter from different farms, using the shallow fermentation method and ammonia injection, did not observe an increase in ammonia concentration in the next batch. Ammonia is a light substance; it volatilizes quickly after removing the plastic tarp from its surface.

Table 2 shows the physicochemical parameters observed in poultry litter treated with ammonia.

Table 2 – Physicochemical parameters measured in poultry litter treated with ammonia gas.

Mensure	Ammonia gas concentration (%)			
	Control	0,25%	0,5%	1%
Temperature (°C)	18,1 [a]	17,35 [a]	17,1 [a]	17,1 [a]
Humidity (%)	21,7 [a]	24,665 [a]	25 [a]	23 [a]
pH	9,25 [a]	9,55 [ab]	9,82 [b]	10,02 [b]
Water activity (aw)	0,880 [a]	0,966 [b]	0,986 [b]	0,999 [b]

Different letters on the same line indicate statistical difference $p<0.05$.

There was no statistical difference (p<0.05) in the parameter's temperature and humidity, in relation to pH and water activity, as the ammonia concentration in the litter was increased, there was a significant increase in these parameters. Ammonia is an alkaline substance (pH 11.6), justifying the increase in poultry litter pH as its concentration increased. The increase in litter pH hinders the growth of microorganisms (Park & Diez-Gonzalez, 2003). In this way, in addition to the microbicidal effect, the continuous use of ammonia in several batches will keep the pH of the litter high, hindering the growth of pathogens such as salmonella. As for the increase in water activity, it is suggested that ammonia, because it is very easy to bind organic matter in the poultry litter, ends up generating more free water. Ghering *et al*. (2020) also observed increased water activity in poultry litter subjected to shallow fermentation.

Thus, the present study may contribute to the improvement of the tarpaulin-on-surface method with ammonia injection in the control of *S.* Heidelberg in reused litters, enabling the production of safer food for the consumer.

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