Research Note: The use of ammonia gas for *Salmonella* control in poultry litters

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ABSTRACT A new poultry litter disinfection methodology against pathogenic *Salmonella* spp. serovars using gaseous ammonia is proposed in this study. In the laboratory, the poultry litter was disposed into plastic containers and experimentally contaminated with 3 *Salmonella* spp. serovars separately. Positive and negative control groups were tested. With a system of hoses, 1% gaseous ammonia was injected into the containers in cycles for 48 h. Samples of the poultry litter were collected and submitted to bacteriological analysis. For the second part, we selected a broiler poultry farm with positive litters for *Salmonella heidelberg* in 2 houses. The littler was treated by gaseous ammonia in a concentration of 2,411 ppm and wrapped with a plastic cover for 48 h during the sanitary break. After the treatment, a new broiler batch was housedand swab samples were collected in the 25-day-old. After the action of the gaseous compound, there was no reisolation of the serovars, and the batches housed on the ammonia-treated litter no longer showed positive results for *Salmonella*. The total elimination of the pathogenic microorganisms by the new method suggests that the controlled use of ammonia gas in poultry litter may represent a viable disinfection technique.

Key words: Ammonia, *Salmonella*, Poultry litter, Broiler

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INTRODUCTION

Poultry litter reuse is a common practice in Brazil because it decreases the production costs and improves the environmental preservation. However, the current techniques used for litter disinfection (shallow fermentation, lime addition, and windrowing) have been showing poor performance, enabling the cross-transmission of microorganisms such as *Salmonella* (Vaz et al., 2017).

The presence of genus *Salmonella* spp. in poultry litter is common because the bacteria naturally inhabits the poultry gastrointestinal tract. Therefore, bacteria are constantly propagated to the environment and may remain viable in poultry litter for long periods (Voss-Rech et al., 2017).

Salmonella typhimurium and *Salmonella* Enteritidis are 2 control target serovars by the Brazilian Federal Inspection Service. According to the current legislation, the entire poultry litter should be removed, fermented, and discarded in the case of positive results. The practice increases the amount of waste in the environment and production costs (Mapa, 2016). *Salmonella heidelberg* raises as a pathogen of worldwide relevance because of the wide prevalence in the poultry production, along with resistance to antimicrobials commonly used to maintain the poultry health. The high frequency of the *S. heidelberg* isolation is possibly due to the greater control over *S. typhimurium* and *S.* Enteritidis by official agencies (Voss-Rech et al., 2015).

Ammonia is a compound naturally involved in several biochemical mechanisms and deployed in industrial sectors. Bird excreta is a large source of ammonia. The gas can cause economical losses such as weight loss and high mortality when in large concentrations during batch development. The compound is widely examined because of its antimicrobial potential against several pathogens (Himathongkham and Riemann, 1999; Koziel et al., 2017). However, ammonia concentrations

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commonly found in poultry litters are insufficient to promote antimicrobial effects (Gehring et al., 2020). To the best of our knowledge, ammonia use in poultry litter against pathogens of genus *Salmonella spp.* is so far poorly investigated.

The objective of this study was to investigate the ammonia gas action against *S.* typhimurium, *S.* Enteritidis, and *S. heidelberg* in experimentally contaminated poultry litter in laboratory and in broiler farms with positive litters for *S. heidelberg*. The hypothesis tested was that ammonia injected in poultry litters under the laboratory condition and in a controlled concentration of 2,411 ppm in a poultry farm during sanitary break can be used to eliminate pathogenic serovars in poultry litters.

MATERIAL AND METHODS

The experiment was performed at the University of Passo Fundo (**UPF**) and in a broiler farm with 2 houses of 150-m length and 16-m width located in the southwest of southern Brazil. This research was approved by the Animal Ethics Commission of the UPF (registry–nº 038/2017). In experiments performed in the laboratory, the strains of *S. heidelberg*, *S.* Enteritidis, and *S. typhimurium* were obtained from the UPF bacteriology laboratory. The poultry litters were collected from a broiler farm located in the northern region of Rio Grande do Sul state, southern Brazil.

The bacteria were stored in the Brain Heart Infusion (**BHI**) broth with 20% glycerol under freezing conditions. For the experiment, they were reactivated in the BHI broth and incubated at 37 ± 1°C for 24 h. Five 10-µL drops of each culture were inoculated into petri dishes containing the Plate Count Agar, and the plates were incubated at 37°C/24 h. The count result was multiplied by 20 and by the dilution factor, obtaining 2.58×10^9 for *S. heidelberg*, 1.79×10^9 for *S. typhimurium*, and 3.25×10^8 for *S.* Enteritidis and it agrees with previous results for *Salmonella* serovars (Voss-Rech, 2017; Vaz et al., 2017). Biochemical identification of *Salmonella* was performed.

The poultry litter (1,000 g) was placed into plastic containers that were adapted for gas injection and detection. A pipe was coupled on one side to connect an ammonia detection sensor (7-NH_3-1000 ammonia/eurogas) and an adjustable valve on the caps to allow injection from a cylinder attached to a hose.

The laboratory tests were performed in quintuplicate for each serovar. Positive control groups (litters contaminated with *Salmonella* and without ammonia action) and negative control groups (litters without *Salmonella* or ammonia action) were also tested. The previously sterilized litters were placed in each container and then contaminated in the laminar flow hood with 1 mL of the BHI broth containing each *Salmonella* serovar separately. Subsequently, they were homogenized in 4 different directions.

The containers were hermetically sealed, leaving a space of approximately 5 cm between the litter and the closure. Ammonia was injected until 1% concentration was reached and left for 48 h. Positive and negative control groups were also tested after 48 h. Samples of the litter (25 g) were collected in 5 points of the container and homogenized in 225 mL of 1% soybean peptone broth with the aid of a mechanical stirrer, remaining in a bacteriological oven at 37 ± 2°C for 24 h. Aliquots of 0.1 mL were taken and transferred to 9.9 mL of Tetrathionate Broth and 1 mL to 9 mL of Rappaport-Vassiliadis broth. The broths were then incubated in a bacteriological oven at 37°C and 42°C for 24 h, respectively. After homogenization, samples were taken with a platinum loop and striated on xylose-lysine-tergitol-4 agar and MacConkey agar for isolation of typical *Salmonella* cultures. Confirmatory biochemical tests were performed in plates where there was growth of typical cultures.

In the poultry farm with 2 positive *S. heidelberg* houses, hoses were laid out under the litter along the whole farm at a lateral distance of 1 m with a 2-mm hole at each linear meter for ammonia gas output. The Aveclean System was used to inject ammonia gas every 4 min per hour, at a pressure of approximately 6 bars during 48 h. At the end of the application the total volume was 320 kg of ammonia. The Aveclean System (Figure 1) distributes ammonia gas, stored in a cylinder, to the entire surface of litter through perforated hoses. To maintain the gas concentration in the litter, a plastic cover was placed to prevent leakage. The injected dose was calculated maintaining the minimum concentration of 1,468 ppm, according to the literature for *Salmonella* elimination in carcasses (Koziel et al., 2017). The application of 320 kg of gas is approximately equivalent to 2,211 ppm, considering the ammonia concentration of 99.5% in 144,000 kg of litter (150-m long × 16-m wide × 0.10-cm high × 600 kg litter/m^3). Owing to possible leakage on the plastic cover and considering the litter heterogeneous thickness, more ammonia was injected than that in a previous study (Koziel et al., 2017). In agreement with the Federal Law of Brazil (Mapa, 2016), 3 samples were collected before the ammonia application and 3 after at the beginning, middle, and end of each house. These samples were composed of 10 subsamples, obtained by the mixture of the litter collected on its surface, in the middle, and near the floor level. Samples were collected 2 h after plastic cover removal. In addition, to confirm the efficiency of the treatment, 4 swabs were collected in the next 25-day-old batch, making 2 samples per house (Mapa, 2016). *Salmonella* isolation was performed using the conventional microbiological method as prescribed previously. The Shapiro–Wilk test was used to test the data normality. Data from the efficiency of ammonia gas against *Salmonella* serovars were statistically analyzed using the Kruskal–Wallis test. Post hoc Dunn's multiple comparison test ($\alpha = 0.05$) was used to identify group differences. Statistical analysis was performed using Statistical Product and Service Solutions (SPSS 23, IBM Corp, Armonk, NY).

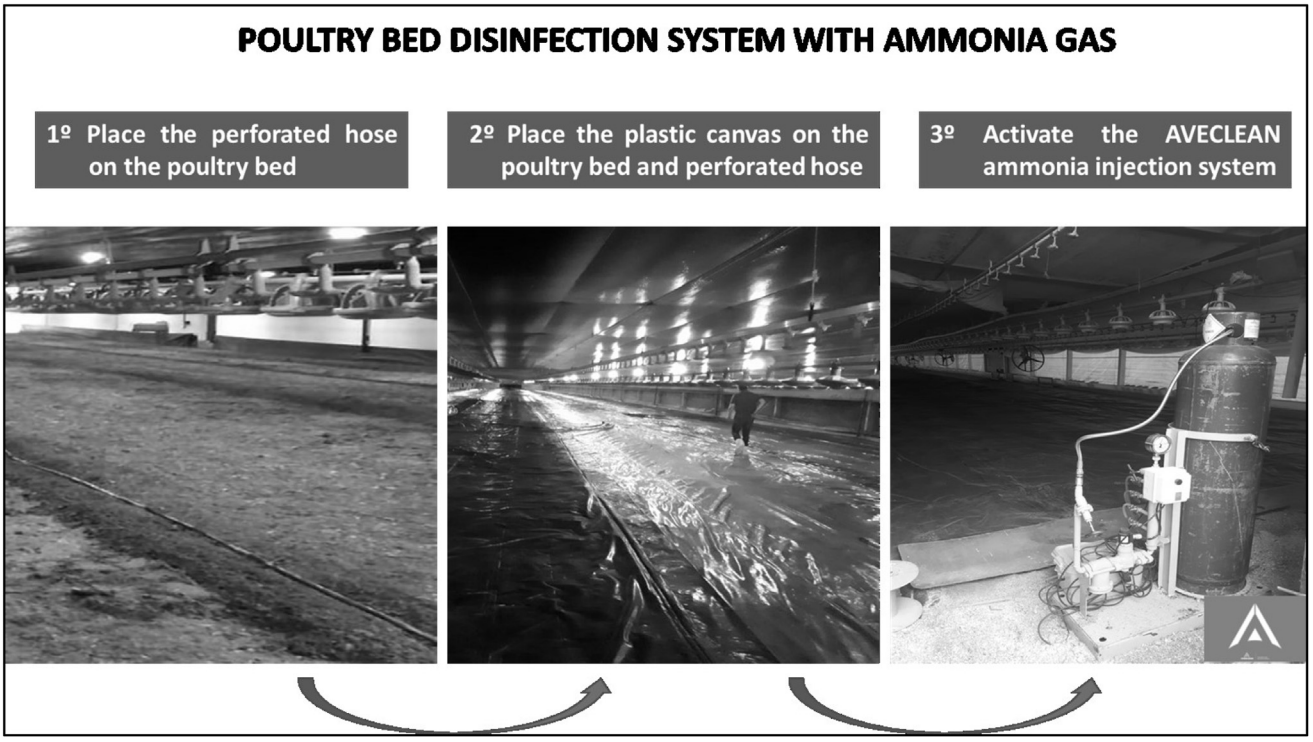

Figure 1. Aveclean system used for ammonia gas application.

RESULTS AND DISCUSSION

Table 1 shows the bacterial growth between groups after ammonia application in the laboratory. The positive control groups exhibited typical pathogen colonies. There was no reisolation of salmonella in the negative group and in the 1% ammonia addition groups. On the poultry farm, there was no isolation of *S. heidelberg* serovar in the litters collected after ammonia treatment and after 25-day-old batch (Table 2).

The tested hypothesis that using 1% ammonia gas in experimentally contaminated poultry litter in the laboratory and applying 2,211 ppm during 48 h in poultry litters positive for *S. heidelberg* would eliminate Salmonella serovars was confirmed, as there was no reisolation of this pathogen in both experiments.

The action of ammonia gas ($\mathbf{NH_3}$) as a disinfectant is associated with the ease that this molecule has to penetrate the cell, increasing the intracellular pH and leading to cell death by electrolyte imbalance (Warren, 1962). In addition to its bactericidal effect against *S. typhimurium* and *Staphylococcus aureus* (Koziel et al., 2017), ammonia gas inactivates several viruses with different genetic backgrounds (Decrey et al., 2016).

Ammonia is naturally produced in poultry litter by bacterial fermentation. Microorganisms transform the uric acid present in bird excreta into ammonia. The reaction speed is controlled by the uricase enzyme, which is dependent of the litter pH (between 8 and 9) and the presence of water and oxygen. The ammonium:ammonia ratio increases at a pH below 7, and the ionized compound (ammonium) does not exert significant bactericidal activity (Warren, 1962). Thus, current litter disinfection methods, such as lime addition, windrowing, and shallow fermentation have been showing reduced efficiency. Lime addition, while increasing the litter pH, cannot maintain a necessary ammonia concentration in litter because of its volatilization. Although the shallow fermentation method and windrowing with plastic cover can retain the ammonia under it, they limit oxygen to microorganisms, reducing bacterial fermentation.

The findings of the present study about *S. typhimurium* agree with previous results (Himathongkham and Riemann, 1999; Koziel et al., 2017). These authors relate the efficiency of ammonia gas against bacteria such as *S. typhimurium*, *Escherichia coli,* and *Listeria monocytogenes.* To the best of our knowledge, there are no reports about the action of ammonia gas against *S.* Enteritidis.

Table 1. Bacterial growth (log.cfu.g^{-1}) between groups (positive and negative control groups and after 48 h of ammonia application).

Salmonella serotype	Positive control ($\bar{x} \pm s$)	Negative control ($\bar{x} \pm s$)	Ammonia 48 h ($\bar{x} \pm s$)	*P*-value
Salmonella Heidelberg	1.75 ± 0.10[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.001
Salmonella Typhimurium	2.45 ± 0.24[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.001
Salmonella Enteritidis	1.85 ± 0.10[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.001

[a,b]The means followed by the same lower case letters on the lines do not differ ($P > 0.05$) by the Dunn test (Kruskal–Wallis post hoc test).

Table 2. Bactericidal effect of ammonia gas on broiler litter that are positive for *Salmonella heidelberg*.

Location of litter collection in the aviary	Before ammonia application	After ammonia application	Swabs in the following lot
Beginning of the aviary (AV1)	*Salmonella heidelberg*	Absent	-
Middle of the aviary (AV1)	*Salmonella heidelberg*	Absent	-
End of the aviary (AV1)	*Salmonella heidelberg*	Absent	-
Beginning of the aviary (AV2)	*Salmonella heidelberg*	Absent	-
Middle of the aviary (AV2)	*Salmonella heidelberg*	Absent	-
End of the aviary (AV2)	*Salmonella heidelberg*	Absent	-
Swab (AV1)	-	-	Absent
Swab (AV1)	-	-	Absent
Swab (AV2)	-	-	Absent
Swab (AV2)	-	-	Absent

Abbreviations: AV1, aviary 1; AV2, aviary 2.

The results of this study diverge from that of Voss-Rech et al., 2017. The authors were unsuccessful in eliminating the pathogen at a concentration of 2,828-ppm ammonium. The result can possibly be explained by the fact that ammonium, as previously explained, does not exert significant antimicrobial effect (Warren, 1962). The concentration and action time of ammonia gas reported in the literature is controversial. There are no studies about a strategical use of ammonia gas in poultry litters (Himathongkham and Riemann, 1999; Decrey, 2016; Koziel, 2017).

Considering the provisions in the Brazilian law (Mapa, 2016), the producers must invest around $1,000 to replace the poultry litter of a 1,200-m^2 poultry farm, besides the loss of days with sanitary break (15 d). Concerns about the destination of these residues to the environment also play an important role in the management of natural resources. The deposition of the litter in the environment also increases environmental microbiological contamination. In slaughterhouses, positive batches for *S. typhimurium* and *S.* Enteritidis are destined to heat treatment. If this process is impracticable, the carcasses should be destined to the production of mechanically separated meat, generating economical losses.

A poultry farmer can carry out a maximum of 7 batches per year, considering the time for broiler growth, slaughter age of 42 d, and 10-day sanitary breaks. With findings of this study on ammonia application, they may reduce the sanitary break up to 4 d, allowing one more batch to be housed. In addition, this practice will benefit potential cost reductions by avoiding the disposal of poultry litter.

Although disinfection methods are different, they all produce ammonia by microbial fermentation or pH elevation. However, several factors are involved in the success of a disinfection method such as the pH level, temperature, humidity, and microbial load. Moreover, the uricase enzyme is also dependent of these factors. Therefore, bacteria elimination is variable in all methods and an alternative could be the controlled addition of ammonia, eliminating the influence of extrinsic factors.

Ammonia is involved in several natural biochemical phenomena and is considered as an indirect generator of the greenhouse effect through the formation of nitrous oxide ($\mathbf{N_2O}$), besides being linked to soil eutrophication and acidification. Ammonia gas emission estimates are still inaccurate (Santana, 2016). Therefore, assessing the environmental impact of large-scale use of an ammonia injection system is needed. However, it must be considered that poultry production and all methods available for disinfection generate ammonia. As this gas production is variable in the broiler farm, there is no exact measure of the released amount involved in the processes. The tested method eliminated *S. heidelberg*, a recognized serovar for resistance. Thus, it is expected that other microorganisms present in the litter and that have no noticeable resistance will also be eliminated, reducing the production of ammonia by bacterial metabolism.

In the present study a new disinfection method was proposed. Our findings show that ammonia gas can be effectively used to disinfect *Salmonella*-contaminated poultry litter. This method reduces the sanitary break from 10 d to 4 d, increasing the profitability of poultry farmers. Although the Aveclean System is efficient and practical, environmental implications were not considered. Therefore, such variables are considered limitations to the present study. This initial research work suggests a trend that requires confirmation by future studies.

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DISCLOSURES

The authors declare no conflicts of interest.

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