MICROBIOLOGY AND FOOD SAFETY

Alphitobius diaperinus control and physicochemical study of poultry litters treated with quicklime and shallow fermentation

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ABSTRACT Poultry litter reuse in Brazil is a common practice to reduce broiler production costs. Quicklime and shallow fermentation treatments are methods used to reduce microbial contamination and infestation of insects such as *Alphitobius diaperinus* (Panzer). The aim of this study was to evaluate the physicochemical parameters of reused poultry litter to better characterize the effects of quicklime and shallow fermentation on *Salmonella* and *A. diaperinus* control. Ammonia and humidity concentrations significantly increased on the litter treated with shallow fermentation and pH when treated with virgin and hydrated quicklime. For *A. diaperinus* control, shallow fermentation with 2 and 3 L of water and 3 L plus 600g of quicklime/m^2 eliminated 100% of the insects. Results of assessed physicochemical parameters indicated that the treatments with quicklime and shallow fermentation are inefficient to control *Salmonella* spp. because they do not reach the indexes required for this pathogen elimination, mainly ammonia and pH. Ammonia index produced by microbial fermentation in shallow fermentation treatment eliminates *A. diaperinus.*

Key words: *Alphitobius diaperinus*, disinfection of poultry litter, broiler and *Salmonella*

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INTRODUCTION

Poultry farming is a featured sector on agroindustry segment. The Brazilian poultry industry has been increasing the number of lots in the same litter with the objective of reducing the production cost in broiler breeding. Poultry litter reutilization is a common practice in many countries, including Brazil. Several bacterial groups compose litter microbiome, influencing poultry litter quality maintenance (Taherparvar et al., 2016) and also are challenges to poultry health and risk to human health, through the consumption of contaminated products (EFSA, 2019). Several poultry litter treatments are used aiming to reduce microbiological risk. In Brazilian poultry farming, the main methods are chemical, quicklime addition, and biological treatments, using fermentative techniques (Vaz et al., 2017).

Quicklime treatment is efficient in control and reduce pathogenic bacteria on the litter, associated with water activity (**AW**) reduction and pH increase, since pH above 9.5 interferes with bacteria survival (McWard and Taylor, 2000; Ferreira et al., 2004). Litter pH can vary from 6 to 9, which allows the multiplication of many relevant bacteria on poultry farming, including pathogens such as *Salmonella* and *Campylobacter* (Jeffrey et al., 2001). Control of mealworm (*Alphitobius diaperinus*) in broiler farms has been increasingly difficult. The use of organic insecticides for a long period has generated resistance (Singh and Johnson, 2015). Mealworm when present in high concentration in broiler farms is considered a reservoir of several pathogens such as *Salmonella* spp. and *Escherichia coli* and may increase the risk of carcass contamination in the slaughterhouse (Crippen et al., 2018). Shallow fermentation method (shallow fermentation of the litter after bird removal) is a latter technique developed in Brazil, which has a superior effect in controlling enterobacteria and insects like *A. diaperinus*, with large ammonia production and lowers costs in reposing sawdust (Silva et al., 2007, 2009). Ammonia when in high levels is extremely important to control pathogens in poultry litter (Voss-Rech et al., 2017). Therefore, the aim of this study was to evaluate the physicochemical parameters of reused poultry litter to better characterize the effects of quicklime and shallow fermentation on *Salmonella* and *A. diaperinus* control.

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MATERIAL AND METHODS

The experiments were performed in a poultry house and in the Research and Diagnostic Center for Animal Health located at the University of Passo Fundo. This research was approved by the Animal Ethics Commission of the University of Passo Fundo (registry–nº 038/2017). Poultry litter was used for 6 flocks. Ammonia and temperature were measured on the poultry house and humidity, water activity, pH, and *A. diaperinus* control were performed in University of Passo Fundo. In the poultry house, 35 squared of litter (1 m^2 each) were delimited. Moreover, 7 treatments with 5 replicates were made: treatment 1 (T1): addition of 600 g of virgin quicklime followed by its incorporation on the litter; treatment 2 (T2): addition of 600 g of hydrated quicklime followed by its incorporation on the litter; treatment 3 (T3): shallow fermentation; treatment 4 (T4): adding 1 L of water to the litter followed by shallow fermentation; treatment 5 (T5): Adding 2 L of water on the litter followed by shallow fermentation; treatment 6 (T6): adding 3 L of water on the litter followed by shallow fermentation; treatment 7 (T7): control group—no treatment was performed on the litter. In treatments that a plastic cover was placed on the litter (T3, T4, T5, and T6), the thickness of the plastic used was 200 microns, and its sides were enveloped in the litter itself to prevent gas leakage. In the center of the plastic cover, a valve was fitted through which the ammonia sensor (7-NH3-1000 AMMONIA/EUROGAS) and the temperature sensor (AKSO) digital dispositive (±1°C precision) were introduced in day 1, 4, and 8. For T1, T2, and T7 treatments, the sensor was placed 01 cm on the litter to measure ammonia. Samples were collected on 1, 4 and 8 D of experiment and forwarded to pH, humidity, and water activity mensuration on lab. Humidity were evaluated by weight difference between samples after drying in 55°C/48 h, and pH were measured diluting 10 g of samples in 50 mL of calcium chloride, homogenizing for 30 min, and using digital pH meter. Water activity were measured using Testo 650 (ITCER-20).

For *A. diaperinus* experiment, 7 treatments with 5 repetitions each were performed: control group, 1 L of water/m^2, 2 L of water/m^2, 3 L of water/m^2, 3 L of water/m^2 with 600 g of quicklime, and 600 g of quicklime/m^2, 600 g of hydrated quicklime/m^2. Poultry litter were put in 35 plastic recipients containing 50 living *A. diaperinus* each. Recipients were closed according to the treatments: for quicklime, hydrated quicklime, and control group, an air passage on the cover was made. A grille was placed to avoid insects escape. For the other treatments (water and water plus quicklime), the cover was placed aiming to emulate quicklime and shallow fermentation methods, respectively. After 7 D, insects were taken, and percentage of living and dead were determined. The ANOVA was performed for the variables AW, pH, humidity, ammonia (NH_3), and temperature, which is based on the decomposition of the total variation of the response variable in plots that can be associated with the treatments (between variance) and the associated experimental error (within variance). The model used was $Y_{ij} = \mu + T_i + E_{ij}$, where Y_{ij} = observation of the ith treatment in the jth experimental unit; μ = overall mean; Ti = treatment effect, and E_{ij} = associated error (Triola, 2014). The Tukey's test was used to compare the treatment means with a significance level of 5% ($\alpha = 0.05$) and significance stated at $P \leq 0.05$. Statistical analysis was performed using SPSS 23 software.

RESULTS AND DISCUSSION

Physicochemical factors develop an important role on microbial inactivation, so your relations with poultry litter microbiome are often studied (Chen et al., 2015; Magri et al., 2015). Results of the experiment are shown in Table 1.

There was no statistical difference ($P > 0.05$) on ammonia concentration between treatments on the different days. Adding water to the litter (T4, T5, and T6) produced more ammonia than T3—treatment without addition. However, the inclusion of 2 and 3 L of water in litter (T5 and T6) generated less ammonia than treatment with addition of 01 L (T4) during the 8 evaluation days. Egute et al., (2010) evaluated ammonia production from the inclusion of water in broiler litter and observed reduced ammonia amounts by adding more than 10 ml/100 gL. This reduction was attributed to the formation of the ammonium ionic compound with water and by the speed reduction of microbial and enzymatic activities because of oxygen excess (Liu et al., 2006). Ammonia antimicrobial effect under *Salmonella* spp. (Park and Diez-Gonzalez, 2003; Islam et al., 2013; Chen et al.,2015) and under viruses is well-known (Ward, 1978; Scodeller et al., 1984; Magri et al., 2015; Decrey et al., 2016), but the mechanism is not yet fully known (Chen et al., 2015). Control group and quicklime addition do not generate detectable ammonia levels. However, observed values on shallow fermentation treatments are inferior to the literature reports (Voss-Rech et al., 2017). In study evaluating ammonium concentration in poultry litter treated under different methods and its effect in *Salmonella* Heidelberg, the pathogen survived over a concentration of 2,828 ppm (Voss-Rech et al., 2017). Kjeldahl method was used to nitrogen detection. However, free ammonia is responsible for the bactericidal effect (Warren, 1962). Gaseous ammonia as antimicrobial is still little studied. Ammonium salts NH_4Cl and $(NH4)_2SO$ were used as a second barrier treatment in chicken carcass digestion process in elevated pH (9), resulting in pathogen elimination after 24 h with 1,468 (Koziel et al., 2017). Ionic force of ammonium allowed to inactive viral endonuclease of foot-and-mouth disease (Scodeller et al., 1984). It was suggested that ammonia leads to a quick alkalinization of bacteria cytoplasm through simple diffusion and proton reducing (Park and Diez-Gonzalez, 2003).

Table 1. Physicochemical parameters measured in poultry litter.

		Shallow fermentation				Quicklime	
Measure	Control	Cover	Cover + 1l	Cover+2l	Cover +3L	Virgin	Hidrated
Ammonia detection PPM							
Day 01	-	421.51^{aA}	550.81^{aA}	585.84^{aA}	533.07^{aA}	-	-
Day 04	-	538.11^{aA}	597.19^{aA}	627.61^{aA}	589.28^{aA}	-	-
Day 08	-	625.94^{aA}	635.66^{aA}	608.38^{aA}	605.24^{aA}	-	-
Temperature (ºC)							
Day 01	26^{aA}	26.48^{aA}	27.58^{aA}	26.62^{aA}	26.80^{aA}	26^{aA}	26^{aA}
Day 04	27^{aA}	27.12^{aA}	27.28^{aA}	27.48^{aA}	27.60^{aA}	27^{aA}	27^{aA}
Day 08	27^{aA}	27.58^{aA}	27.26^{aA}	27.46^{aA}	27.46^{aA}	27^{aA}	27^{aA}
pH							
Day 01	7.98^{aA}	7.90^{aA}	8.13^{aA}	8.03^{aA}	8.24^{aA}	8.32^{aA}	8.38^{aA}
Day 04	8.04^{aA}	8.09^{aA}	8.36^{aAB}	8.35^{abA}	8.31^{aAB}	8.56^{aA}	8.69^{aAB}
Day 08	7.97^{aA}	8.11^{aBC}	8.25^{aABC}	8.16^{aABC}	7.99^{aC}	8.73^{aA}	8.65^{aAB}
Humidity							
Day 01	25.77^{aA}	27.75^{aA}	35.38^{aB}	36.09^{aB}	37.59^{aB}	31.61^{aB}	33.29^{aB}
Day 04	18.07^{bA}	28.59^{aA}	38.04^{aB}	38.06^{aB}	41.04^{aB}	19.27^{bA}	21.01^{bA}
Day 08	15.54^{bA}	35.15^{bB}	41.11^{bB}	58.63^{bC}	69.00^{bD}	13.96^{cA}	16.04^{cA}
Water activity (aw)							
Day 01	0.96^{aA}	0.94^{aA}	0.95^{aA}	0.94^{Aa}	0.96^{aA}	0.94^{aA}	0.95^{aA}
Day 04	0.90^{aA}	0.94^{aABC}	0.96^{aAC}	0.97^{Aac}	0.97^{aAC}	0.90^{aA}	0.88^{abAB}
Day 08	0.80^{bAB}	0.97^{aAC}	0.96^{aAC}	0.98^{Ac}	0.99^{aC}	0.89^{aA}	0.85^{bAB}

Different lowercase letters indicate statistical difference between a column ($P<0.05$). Different uppercase letters indicate statistical difference between lines ($P< 0.05$).

There was no statistical difference on temperature parameter between treatments and between the days evaluated. Values did not reach valid levels to microorganisms inactivation (Macklin et al., 2006; Guan et al., 2009) and in *A. diaperinus* mortality, since values founded are proper to *A. diaperinus* cycle of development (Rezende, 2010). Leaning treatment method allows to reach high temperature values. Also, in this method, lower microbial contamination and high mortality indexes of *A. diaperinus* was observed (Flores et al., 2009). Fermentative methods reached 60°C; however, there are limitations to reach this value evenly (Fiorentin, 2006). *Salmonella* spp. can survive in the environment for long periods, up to 18 mo in poultry litter (Williams and Benson, 1978), extreme temperatures (2°C–54°C), and in low humidity (Andino and Hanning, 2015). Therefore, quicklime and shallow fermentation methods do not generate enough temperature increase to eliminate microorganisms and insects such as *A. diaperinus*.

The addition of virgin quicklime and hydrated quicklime in the litter (T6 and T7) increased the litter pH compared with the other treatments. However, no treatment has shown pH higher than 9, which is necessary to inactivate microorganisms (Park and Diez-Gonzalez, 2003). Virgin quicklime has dry action, and the antimicrobial activity is due available water reduction associated with increase in poultry litter pH (Ruiz et al., 2008).

Application of quicklime in poultry litter for the same time of this experiment in lower concentration resulted in pH 9.6, which does not eliminate *Salmonella enteritidis* (Vaz et al., 2017). In sludge treatment, when virgin quicklime is used for microbial reduction, pH 12/2 hs is needed to eliminate pathogens (USEPA, 1999). However, virgin quicklime concentration in poultry litter does not make pH reach higher values than 9 (Cassity-Duffey et al., 2015) . The elevation of pH, by adding quicklime or plaster, may have beneficial actions in reducing bacteria concentration (Burgess et al., 1998).

Studies reported that reused poultry litter showed significantly higher pH and volatilized ammonia values when compared with first use litter. This can be explained by reused litter having more uric acid, which increases these physicochemical parameters (Traldi et al., 2007). Reutilization of virgin and hydrated quicklime on litter helps to elevate pH, which improve uricase enzyme production that leads do ammonia production (Kim and Patterson, 2003). Ammonia levels are insignificant when pH is lower than 7 (Reece et al., 1980), because of higher ammonium production (Payne et al., 2007) and rise significantly when pH is over than 8 (Trabulsi and Alterthum, 2008). This can explain why quicklime addition and shallow fermentation for litter treatment has low efficiency in litter disinfection on first flocks. First-use litter has a pH value of 6, and low manure quantity is insufficient to provide fermentation and ammonia production.

Table 2. *Alphitobius diaperinus* mortality in poultry litter submitted to conventional poultry litter treatment methods.

Treatments	Dead insects (%)
Control	39.33^{a}
Virgin quicklime	58.00^{ab}
Hydrated quicklime	68.80^{b}
1 L/m^2	24.40^{a}
2 L/m^2	100.00^{c}
3 L/m^2	100.00^{c}
3 L/m^2 + 600 g quicklime	100.00^{c}

Different lowercase letters indicate statistical difference between a column ($P < 0.05$). ANOVA and Tukey's test were made only in control group, virgin quicklime, hydrated quicklime, and 1 L/m^2 treatments.

Treatments with shallow fermentation showed higher humidity when compared with the ones treated with quicklime and control group. Quicklime catches litter humidity, whereas shallow fermentation isolate litter, not allowing water produced by bacteria fermentative processes to volatize. One of the main factors to increase microbial proliferation is the humidity, raising fermentation and gas liberation, such as nitrates, ammonia, and hydrogen sulfate (McWard and Taylor, 2000). Microorganism's dynamic equilibrium depends of adapting to the environment, determining competitivity (Correa et al., 2000). *Salmonella* survival was often observed in higher humidity poultry litter, and this factor reduced bacteria population (Opara et al., 1992; Islam et al., 2013). Humidity is one of the main factors in ammonia emission. So, controlling this parameter is vital to avoid ammonia issues in poultry houses (Ritz et al., 2004). However, ammonia volatilization in poultry litter with high humidity is reduced because of the intense dissociative effect of ammonia in water (Medeiros et al., 2008) and due the fact that microbial and enzymatic activities are reduced or ceased because of oxygen scarcity (Trabulsi and Alterthum, 2008). In poultry litter with less humidity indexes, urea conversion in ammonia might be reduced (Trabulsi and Alterthum, 2008). In this study, it no significant increase of ammonia was observed when different volumes of water were added.

The medium values of water activity did not show statistical difference, except on eighth day (0.99). The results found on every treatments helps microorganism's growth. Water activity of poultry litter commonly reach 0,9 indexes (Fiorentin, 2005). Studies about water activity indicated *A. diaperinus* mortality with 0.94. Water activity has been applied on poultry farming to control *Salmonella* sp. (Opara et al., 1992) and *E. coli* (Flores et al., 2009). Relation between pH and water activity in reduction of *Salmonella* population on litter was AW $\leq$ 0.84 and pH $\leq$ 4.0 (Payne et al., 2007) Also, quicklime addition reduce water activity in poultry litter (0.88 – 0.85), leading to higher effort on microbial survival (Hills et al., 1997). Thus, the physicochemical parameters evaluated in this study can be an alternative to an efficient poultry litter treatment. However, ammonia levels on conventional methods are not enough to eliminate pathogens. Studies show that shallow fermentation method is better than quicklime addition. This can be explained mainly because of the fact that ammonia is held under the cover, increasing concentration, whereas in quicklime treatments occurs a volatilization of the compound, reducing its efficiency (Table 2). Recent unpublished studies at the University of Passo Fundo demonstrated that injecting 10,000 ppm of ammonia gas under the shallow fermentation litter eliminated *Salmonella enteritidis*, *Salmonella typhimurium*, and *S.* Heidelberg within 48 h of application. Therefore, shallow fermentation treatment with ammonia gas emanates as an economical, practical, and inexpensive method for *Salmonella* elimination in contaminated poultry litter "(verbal communication)".

Treatments with 2 and 3 L/m^2 and 3 L + 600 g of quicklime led to 100% mortality of *A. diaperinus*, probably because of higher ammonia production. Treatment with 01 L/m^2 showed no statistical difference when compared with virgin quicklime and control group treatments. Also, in every repetition of this treatment, the insects were found on the bottom of the plastic recipients. This occurred probably because of ammonia volatility, which is concentrated in the upper portion of the recipients. In mortality parameter in the control group, the probable cause was the lack of humidity in the litter and the fact that all insects were adults, probably on the end of life cycle.

There was no statistical difference between virgin quicklime and hydrated quicklime. However, hydrated quicklime treatment had higher mortality indexes when compared with virgin quicklime, probably because of the higher pH of the first one. Quicklime used in poultry litter elevates pH and low free water content, therefore lowering humidity (Ferreira et al., 2004). It was suggested that shallow fermentation method between flocks reduces residual contamination of adult insects and maggots in poultry litter because of ammonia toxicity. In the experiment, the referred method showed temperature between 23.8°C and 32.1°C, which did not influence the mortality of insects (Rezende, 2010). In ammonia volatilization, a progressive increase was observed in 3 measurement moments: 4, 8, and 12 D, which may have influenced mortality of insects (Rezende, 2010). Unlike mammals, insects perform gas exchange through tracheal tubules that connects to their body cells. Ammonia inhalation reaches these cells, raising intracellular pH. Recent unpublished studies from University of Passo Fundo "(verbal communication)" demonstrated that maggots and adults of *A. diaperinus* exposed to 1% gaseous ammonia died in less than an hour.

From the physicochemical studied parameters, there was an increase in ammonia concentration in the shallow fermentation treatment and in pH in the quicklime addition treatment. However, the detected ammonia and pH levels are not enough to eliminate pathogens such as Salmonella. For mealworm control in containers sealed with canvas, adding 02 and 03 L of water and 03 L of water plus 600 g of quicklime per m^2 eliminated 100% of the insects.

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