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Manure Solarization from Cattle, Goat and Poultry and its Effect on Survival of *Salmonella* spp

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Application of raw livestock manure in agricultural practice could potentially cause contamination of foodstuffs with pathogenic bacteria such as *Salmonella* spp. In this study, manure from cattle, goat and poultry were solarized during eight weeks in order to evaluate the *Salmonella* survival. Six piles of each type of manure were cover with transparent plastic and half of the treatments were added with 50% of water basis on manure dry weight. *Salmonella* was detected previous to solarization process in the three manure types and at the last week was not detected at neither with and without water addition treatments by microbiological and PCR detection. Treatments with water addition reached the highest temperatures and had the lower log10 CFU g^{-1} level of *Salmonella*. The solarization process by itself can be effective in the reduction of pathogens level, however, the use of manure with some water content can improve the eliminating pathogens process. Thus, properly solarized manure can be safely used in food crop production while eliminating the likelihood of microbial contamination.

Key words: Livestock manure, solarization, manure-water content, *Salmonella* survival.

In organic agriculture, animal manure application to the soil is unquestionably necessary because is important to renew the soil organic matter and nutrient supply; however, the application without prior treatment to destroy pathogens increases the risk of microbiological contamination of agricultural products[1,2]. Globally, large quantities of manure are produced continuously. In Mexico annually is produced 61 million tons of manure dry basis of different animal species, representing the cattle manure the 83%, and the Comarca Lagunera, a region where there is a large livestock activities, produce about 925 000 tons of manure dry basis yearly[3,4]. This intensive production of animal manure,generates waste of high contents in nutrients and organic material that contaminate soil and water, emit unpleasant odors and generates gases in addition to promoting the proliferation of vectors and pathogens, all with a negative impact in the environment and human health[5,6]. Particularly, there is a significant health risk, which is given by the presence of pathogens such as *Salmonella* spp., which may be present in animal manures.The survival of pathogens in manure depends on many factors, including temperature, humidity, pH, physical composition of the composting materials, the type of manure, and the microbial competition[7,8]. The presence of *Salmonella* and other pathogens in the manure has prompted to seek methods of manure treatment before application through composting, pasteurization, steam drying, or UV radiation in order to reduce the amount of fecal coliform bacteria and helminth eggs[9].One method that has been successfully used in agricultural soil disinfection

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is solarization which is an hydrothermal technique of pathogens removal that involves covering the manurefor several weeks with a polyethylene film that has the ability to capture solar radiation and increase the temperature significantly causing physical, chemical and biological effects[10,11,12]. The effect of solarization on the elimination of pathogens is mainly due to thermal inactivation due to the high temperatures reached[13]. In this regard, the goal of this study was to evaluate the use of plastic without albedo on the solarization of three animal manures (cattle, goat and poultry) with addition of water, as an active disinfecting method for removing *Salmonella* in the manure and comply it with current regulations for use as safe organic fertilizer.

MATERIALS AND METHODS

Geographic location

This experiment was carried out in the region known as the Comarca Lagunera, located in the central part of the northern Mexico, in the states of Coahuila and Durango, between the meridians 102° 22'and 104° 47' west longitude and the parallels 24° 22'and 26° 23' north latitude, with an average height above sea level of 1,139 m. The climate of the region is classified as steppe (BS) and desert (BW), with rains in summer and cold winters. The average annual rainfall is 230 mm and evaporation of 6-11 times higher than precipitation. The average annual temperature is 22 °C, reaching 42 °C in summer and 4 °C in winter. The relative humidity in the region varies according to the season, with 31% in spring, 47% in summer, 58% in autumn and 40% in winter.

Experimental site and preparing of manure piles

The study was conducted in the period from August 31 to October 20 of 2011 in the experimental field of the Facultad de Agricultura y Zootecnia, located at kilometer 28.5 of Tlahualilo-Gomez Palacio road. The three types of manures were collected at farms located at the Durango state, Mexico. Manure piles were constructed of 2 m long, 1 m wide and 1 m high. To carry out the process of solarization manure piles were covered with transparent plastic without albedo (manufactured by Plastoza, SA, State of Mexico) PLANAT type 180 x 1000/100 (1.8 m wide, 1,000 m long) and 100 ì thick.

Treatments evaluated

The treatments were: T1 = cattle manure pile, T2 = cattle manure pile plus 50% more moisture, T3 = goat manure pile, T4 = goat manure pileplus 50% more moisture, T5 = poultry manure pile, T6 = poultry manure pile plus50% more moisture. The experimental design was a randomized block with three replications and factorial arrangement 3x2, being the factor A = manure types (cattle, goat, poultry), factor B = moisture levels (original moisture, 50% of water added). All piles were covered with a single plastic cover. In treatments with more moisture, 50% of water was added based on manure dry weight and turned manually with shovels for homogenization. In each pile of manure, temperature was measured daily at the depths of 15 and 30 cm for 51 days. Temperatures were measured with a direct temperature meter (HANNA HI-99 121, Spain).

Samples of manure

For each type of manure prior to solarize a sample of 40 g was obtained for microbiological and molecular initials analysis. For further analysis during the time of solarized, every 12 days, a composite sample of 20 g were taken at depths 0-15 cm and 15-30 cm respectively.The samples were placed in Ziploc plastic bags 15x30 cm, to transfer and immediate microbiological analysis.

Enumeration of *Salmonella*

From each composite manure sample 2 g was aseptically transferred to a sterile centrifuge tube of 50 ml, were added 18 ml of sterile physiological saline solution (PSS) (0.85% NaCl, Sigma Aldrich, USA), and the mixture was vortexed for 6 min (Maxi Mix Plus, BI, USA). The extract was diluted in series (1:10) with sterile PSS, and 0.1 ml aliquots were plated onto brilliant green agar with sulfadiazine (BGS) (Becton Dickinson and Co. Mexico). After incubation at 35 ± 2 °C for 18-48 h colonies meeting*Salmonella* characteristics were counted.

DNA extraction

From each composite manure sample, 1 g was aseptically suspended in 9 ml of buffered peptone water (BPW) (Becton Dickinson and Co. Mexico) and incubated at 35 ± 2 °C for 24 hours. After this time, 1 ml was transferred in 9 ml of tetrathionate broth (TT) (Becton Dickinson and Co. Mexico) and incubated at 42 ± 0.5 °C for 24 h. From TT broth, DNA extraction was done with the

CTAB method (cetyltrimethyl ammonium bromide) but omitting the use of polyvinylpyrrolidone and mercaptoethanol[14].The extracted DNA was stored at "20 °C.

PCR reactions for *Salmonella* spp

The presence of *Salmonella* was also diagnosed by PCR, based on the *invA* gene. The PCR mixture contained 25 pmoles of each of the following primers[15](F 5 'GTGAAATTATCGCCA CGTTCGGGCAA 3';R 5 'TCATCGCACCGTC AAAGGAACC 3'), 200 ìM of each of the 4 deoxynucleoside triphosphates (Bioline Inc.,USA), 1 mM MgCl2, 1× Reaction Buffer (200 mMTris-HCl pH 8, 500mMKCl), 2.5 U of *Taq* DNA polymerase (Promega, USA.), 100 ng of DNA template, and deionized water for a final volume of 25 ìL. PCR reactions were done in a thermal cycler Model TECHNE TC512 (Barlo World Scientific, USA).The reaction mixture was subjected to the following thermal cycling conditions: heat denaturation at 95 °C for 1 min, and then 35 cycles with heat denaturation at 95 °C for 30 s, primer annealing at 58 °C for 30 s, and DNA extension at 72 °C for 30 s. After the last cycle, samples were maintained at 72 °C for 10 min to complete synthesis of all strands. The PCR product(287 bp) was subjected to gel electrophoresis (1.5% agarose) (Promega, USA), and then stained with ethidium bromide (0.5 ìg/ mL) (Sigma Aldrich, USA), visualized with a UV transilluminator (Spectroline Transilluminator, Model 7C-254R. Electronics Corp., USA.), and the images were captured with the gel documentation system WiseDocWGD-20 (DAIHAN Scientific Co. Ltd. Korea).

Statistical Analysis

One-Way ANOVA and mean separation tests (LSD, P≤ 0.05) were performed with SAS statistical software[16].The variables were the temperature and the CFU g^{-1} of manure, transformed to $\log_{10}$.

RESULTS

Temperature behavior in treatments

Significant differences ($p<0.01$) was found in the temperature record for the factors manure types, moisture level, sampling depth, sampling date, as well as the interactions type of manure with moisture level, type of manure with sampling date, moisture level with sampling date, sampling depth with sampling date and type of manure with moisture level and sampling date.The average values of temperature throughout the study period bymanure types, moisture level and sampling depth are shown in Table 1. The poultry manure showed the highest average value of temperature ($p<0.05$), followed by cattle and goat manure, with a difference of 1.86 °C over the lowest average value (goat manure). Concerning to the effect of adding water to the treatment or not, the highest average value was observed in those treatments to which water was added ($p <0.05$) with a difference of 2.35 °C with respect to which no water wasadded. In relation to the depth of sampling, the average highest temperature was observed in the depth of 15-30 cm ($p <0.05$) with a difference of 3.17 °C with respect to the depth of 0-15 cm.

The maximum temperatures recorded in the study by manure types, with or without the addition of water and sampling depth are shown in Figure 1. Regardless of manure types, the highest temperatures were observed in treatments with added water and in the depth of 15-30 cm. The maximum temperature recorded in the poultry manure was 63.37 °C, in bovine manure 62.35 °C and goat manure 60.43 °C, respectively with water addition at the depth of 15-30 cm. The minimum temperature recorded in goat manure was 47.67 °C, in bovine manure 47.86 °C and in the poultry manure 50.0 °C, respectively without water addition

Table 1. Comparison of mean temperatures for manure types, moisture level and depth of sampling

Manure types	Mean	Standard deviation	Moisture level	Mean	Standard deviation	Sampling depth	Mean	Standard deviation
Poultry	47.60 A	3.65	50%	47.54 A	5.68	15-30	47.95 A	5.92
Cattle	45.77 B	4.00	0.0%	45.19 B	5.77	0-15	44.78 B	5.33
Goat	45.74 B	4.17						
LSD	0.41			0.33			0.33	

Values with different letters are significantly different ($P < 0.05$).

Table 2. Comparisonof means of CFU (log_{10}cfu g^{-1}) for manure types, moisture level and sampling number

Manure types	Mean	Standard deviation	Moisture level	Mean	Standard deviation	Sampling depth	Mean	Standard deviation
Goat	2.76a	0.61	0.0% added.	2.63 a	0.43	Preliminary	3.02 a	0.30
Cattle	2.49b	0.43	50.0% added.	2.48 b	0.58	1	2.86 b	0.28
Poultry	2.42b	0.42				2	2.47 c	0.11
						3	1.85 d	0.27
						4	0.00 e	0.00
LSD	0.07		LSD	0.05		LSD	0.08	

Values with different letters are significantly different (P< 0.05).

at the depth of 0-15 cm. The maximum temperatures recorded at the two depths were different, being higher in the depth of 15-30 cm with a range from 53.37 to 63.37 °Cand an average difference of 9.0 °C compared to those recorded in the depth of 0-15 cm with a range of 47.67 to 51.95 °C.

Figure 2 shows the average maximum and minimum temperatures recorded at two depths in eight dates across the study period, showing almost constant behavior. The average minimum temperature at both depths was 30 ° C in the last sampling date (day 51), while the average high temperature in the depth of 0-15 cm was 60 °C at date 1 (day 6) , and in the depth of 15-30 cm was 62 °C at date 3 (day 18). In general, the minimum and maximum average temperatures were relatively lower on the last date (day 51).

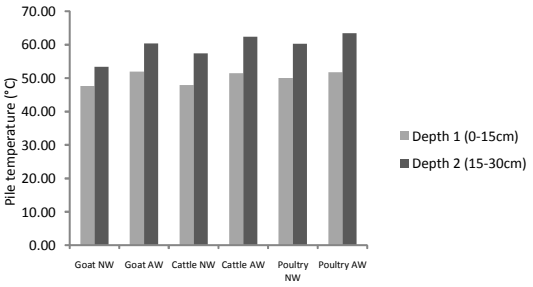

Fig. 1. Maximum temperatures recorded by manure types, with (AW) and without (NW) addition of water in two depths.

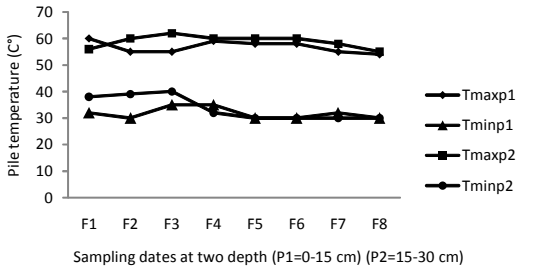

Fig. 2. Average temperatures maximum and minimum recorded at eight dates and two depths through the study period

***Salmonella* CFU behavior**

Significant differences ($p<0.01$) were found in the CFUfor factors manure types, moisture level, date of sampling and the interaction manure types with sampling date. Microbiological analyses were not performed for sampling depth. Table 2 shows the mean values of CFU (log_{10}cfu g^{-1}) in the three manuretypes, moisture levels and sampling number. No significant differences ($p = 0.05$) were observed between the mean values of cattle manure and poultry manure, while the goat manure was the one with the highest average value of CFU ($p<0.05$). In relation with and without the water addition, the treatments where water was added had the lowest average value ($p<0.05$).

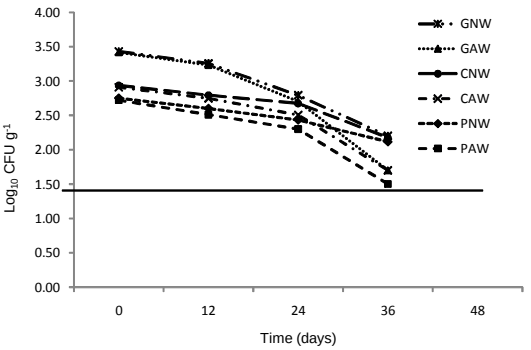

Fig. 3. *Salmonella* spp survival in: goat manure without addition of water (GNW) and with addition of water (GAW), cattle manure without addition of water (CNW) and with addition of water (CAW), poultry manure without addition of water (PNW) and with addition of water (PAW). Solid line indicates that the organism was not detectable by enrichment culture

Regarding the number of sampling, all were different ($p<0.05$), with an average reduction rate of 1.6 $\log_{10}$ CFU g^{-1} to the level of no direct detection in plate. The rate of reduction of *Salmonella*during solarization in the three types of manure appears to be strongly dependent on the temperature and water addition.

Survival of *Salmonella*

CFU concentrations by manure types, sampling date, with or without the addition of water, and before and during treatment of solarization are shown in Figure 3. Higher initial concentrations were observed in goat, cattle and poultry manure in that order. Respect with and without water addition, in the treatments with water addition the number of CFU was lower than in the treatments without water addition and this trend was observed for the three manure types.According to figures 1 and 3 a negative relationship was observed between the number of CFU with the temperature and addition of water to the treatments. In the different sampling during the solarization, the three manure typesshowed the same tendency in the reduction number of CFU, being undetectable in plate in the fourth sampling (day 48) in any of the three manure types.

Detection of *Salmonella* by PCR

Figure 4A shows the results of PCR for the *invA* gene of *Salmonella* before solarization process of manure. It can be seen that in the three samples of the three manure types, was amplified the expected band of the PCR product (287 base pairs) and therefore is deduced that were positive for the presence of *Salmonella*. 4B shows the PCR results of the last sampling date (day 48), showing that no PCR products were amplified for any of the samples indicating that *Salmonella* was not present, except for the positive control in which the PCR product was amplified.

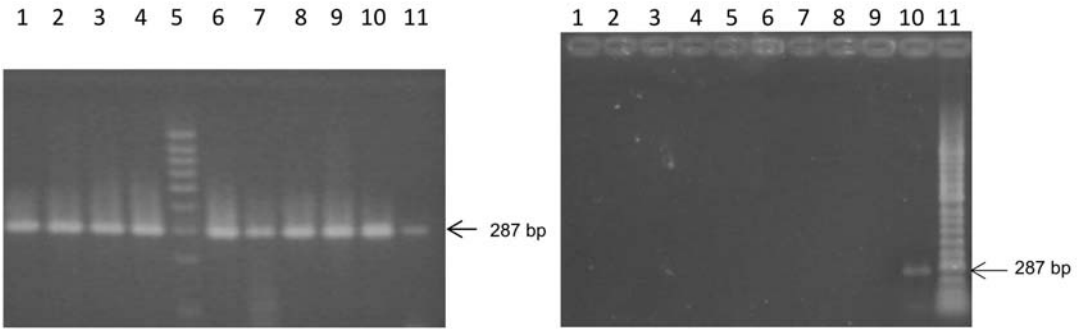

Fig. 4. Amplification of *invA* gene of *Salmonella* from different manure types before (4A) and after (4B) solarization process.4A. Lanes 1-3, goat manure samples, 4, 6 and 7 cattle manure samples, 8-10, poultry manure samples, 11, positive control *S.* Typhimurium, 5, 100-bp ladder (Bioline, USA). 4B. Same samples on the last sampling date (day 48). Lane 10, positive control *S.* Typhimurium, lane 11, 50-bp ladder (Bioline, USA).

DISCUSSION

In rural areas the manure from different animal species accumulates in large quantities, contaminating soil, surface water, groundwater, and air. In most cases these manures are used as organic fertilizers in agricultural production and commonly are the introduction way of pathogens through the food chain[17,18]. Therefore, the manure before being used as organic fertilizer should be treated with any active method like solarization, with the intention of eliminating pathogens of plants, animals and humans[10, 12].In this study, the three manure typesshowed differences in the temperature reached during the solarization, which could be due to initial differences of microbial load and therefore of microbial activity, factors contributing to the warming of the manure[19]. In general temperatures were higher with the addition of water to the treatments and in the depth of 15-30 cm. The water level added (50%) was within the limits considered as optimal for active composting[20] and contributed to the temperature was higher in treatments with water added, being this effect

reported as normal when manure is wet[21].

The temperature differential in the range observed at each depth, as well as the differential between the two depths (figure 1) may be due to the fact of have been uncovering the manure piles to measure the temperatures daily and to sampling manure every 12 days for microbiological analyzes,however, spite of the above, conditions were promoted to achieve the minimum temperatures necessary for effective solarization. In a study was showed in a scale composting system that *Salmonella*tends to disappear in 48 h with a temperature of 45 °C, while *E. coli* O157: H7 did it in 72 h[22]. Other study reported the removal of *E. coli* in feedlot cattle manure in the first 7 days of composting when the average temperatures of the composting rows were 33.5 to 41.5 °C[23].

Average temperatures for eight record dates distributed throughout the study period showed almost a constant behavior (Figure 2) which reflected a high microbial activity throughout the period[19]. This aspect to maintain constant temperatures throughout the period of solarization and above the maximum tolerated by the pathogen under study is important because it ensures the elimination. In the United States, according to the EPA regulations for efficient composting of biosolids should consider either a minimum temperature of 55 °C for 3 days in aerated static piles or 15 days with five turned by the biosolids in the row systems[24].In this study, manure piles were not turning, as is usually done in the process of composting, however, have uncovered the piles daily (about 5 min) to check temperature and every 12 days to take manure samples for microbiological analyzes, allowed the entry of oxygen. Oxygen allows aerobic bacteria degrade organic matter in the pile, generating heat and the internal temperature keeps for a longer period of time, as in the present study[25].

The survival of pathogens in manure depends on many factors, including temperature, humidity, pH, physical composition of the composting materials, waste type, and microbial competition[7, 8] The three manure types differ ($p<0.05$) in the initial concentration of *Salmonella*, being this concentration higher in goat manure (Figure 3). In a study were found differences in *Salmonella* survival rate between fresh and old poultry manure, mentioning that were due to differences in the kinetics of heat transfer due to the variation of the composition manure and physical properties, including moisture content and particle size[26].

Differences were observed ($p<0.05$) in the number of CFU between treatments with and without addition of water, been concentration lower in treatments with added water. From the relationship between the CFU and the treatments with and without addition of water, it shows a negative relationship between higher temperature and lower number of CFU in treatments with added water.Thermal resistance of microorganisms increases with decreasing moisture content[27], likewise, as the cell is heated, the water molecules begin to vibrate and this vibration breaks the disulfide bonds and hydrogen bonds in proteins, which may alter the three-dimensional configurations and prevent the correct protein function. The lower the amounts of water present, these vibrations are reduced, lowering the protein denaturation by this mechanism, increasing the resistance of the microorganisms[26].In a study of composting, temperatures were 60 °C for three weeks, enough to kill *E. coli* and *Salmonella*, however, there is the possibility of re-growth of the population not detectable by the heterogeneity of composting in the piles[28]; Other studies with solarization determined that the temperature which inactivates *Salmonella*enteritidis PT4 occurs at 60 °C[29, 30].

The results of the CFU of the four samples in the three manures, showed a significant decrease ($p<0.05$) in the level of *Salmonella* in the course of time until the level of no direct detection in plate (figure 3), which shows the favorable effect of solarization. It has been mentioned that with a minimum effective time of 20 days of solarization of cattle manure, is sufficient to eliminate the presence of *Salmonella* and *E. coli*[31].The presence of *Salmonella* in composted products leads to the hypothesis of failures in the composting process[32]a situation that was not presented in this study, because in the final sampling was not detected the presence of *Salmonella*, complying with the definition of ecological quality of the products of composting that was established in the Decision of the European Commission[33].

Initial sampling of manure before starting the process of solarization, indicates the presence

of *Salmonella* by PCR, while in the last sampling the results were negative. Is known that nucleases destroy free nucleic acids[34] which may explain the absence of *Salmonella* DNA in the samples and therefore the negative PCR results in the last sampling.It has been noted that when the temperature of the compost microcosm rose to 50 °C or above, neither the plasmids and their hosts *E. coli* could be detected, concluding that composting temperatures above 50 °C can be expected to destroy plasmids[35]. Being plasmids circular DNA molecules, the results suggested that the high temperatures generated during composting of chicken manure may help prevent the spread of antibiotic resistant genes through plasmids into the environment.

CONCLUSIONS

We demonstrated that thermophilic solarization of manure plus addition of 50% of water (dry weight basis) is one active method for biological control of pathogens under conditions which allow for the development of high temperatures (53 to 63°C) resulting from heat diffusion biologically produced. The final manure can be freeof pathogens or has substantially fewer pathogens than the original manure, depending on the adequate plastic cover, correct application, time duration and adequate moisture content of manure among other influencing factors.

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