BORNH Bulletin of Regional Natural History
Formerly Bollettino della Società dei Naturalisti in Napoli

Survival of *Salmonella* Typhimurium, *Escherichia coli*, and *Enterococcus faecalis* in poultry manure samples, treated with different concentrations of hydrated calcium hydroxide

Federica Carraturo[1]*, Michela Morelli[1], Marisa Di Santo[2], Patrizia Ambrosino[2], Teresa Crovella[2] and Marco Guida[1]

DOI https://doi.org/10.6093/2724-4393/8118

***Correspondence:**
federica.carraturo@unina.it
https://orcid.org/0000-0003-0359-3297

Affiliation:
[1] Department of Biology, University of Naples Federico II, Naples, Italy. Meridionale, Naples, Italy.
[2] 2AGRIGES srl - Nutrizione Speciale per L'Agricoltura Biologica e Integrata, San Salvatore Telesino (BN), Italy.

Conflict of Interest: The authors declare that they have no conflict of interest.

Financial Disclosure Statement: The Authors declare that no specific funding was received for this work.

Accepted: 29 June 2021

Abstract

The aim of the study was to verify the quality and microbiological safety of poultry manure, a completely natural fertilizer, composed of the manure of hens, poultry, and other birds. The evaluation of the quality and safety of poultry manure was performed following a bactericidal treatment, using different percentages of hydrated calcium hydroxide, inhibiting the growth, proliferation, and survival of bacterial species that can be pathogenic for humans. The evaluations were conducted spiking known concentrations of *Salmonella* spp., *Escherichia coli*, and *Enterococcus* spp. Following the contamination, qualitative and quantitative analysis for the research of the above-mentioned pathogens were performed. In parallel, the variations of pH and humidity in the samples under examination were verified. The experiments consisted on adding different concentrations of hydrated calcium hydroxide, with a percentage ranging from 15% to 25%, followed by the qualitative and quantitative research of the pathogenic microorganisms spiked in increasing ten-fold concentrations (10^2, 10^3, 10^6 CFU/g). The total reduction of the microbial load in a period of time that varies from a few hours to a few days, depending on the microorganism under

consideration, was observed.

Keywords: Hydrated calcium hydroxide, poultry manure, microbial reduction, *Salmonella* spp., *Escherichia coli, Enterococcus* spp.

Riassunto

Lo scopo della sperimentazione è stato quello di verificare la qualità e la sicurezza microbiologica della pollina, un fertilizzante completamente naturale, costituito da letame di galline, polli ed altri volatili.
La valutazione della qualità e sicurezza della pollina è stata valutata a seguito di un trattamento battericida, ottenuto utilizzando diverse percentuali di idrossido di calcio idrato (o, calce idrata), in grado di inibire la crescita, la proliferazione e la sopravvivenza di specie batteriche che risultano patogene per l'uomo.
I test sono stati condotti mediante l'inoculazione artificiale di concentrazioni note di *Salmonella* spp., *Escherichia coli*, ed *Enterococcus* spp. Successivamente alla contaminazione, sono state condotte analisi qualitative e quantitative al fine di ricercare i patogeni sopraindicati. In parallelo sono state analizzate le variazioni del pH e dell'umidità nei campioni in esame.
La sperimentazione è consistita dunque nell'aggiunta di diverse concentrazioni di calce idrata, con concentrazioni d'uso comprese nel range 15-25%, seguita dalla ricerca qualitativa e quantitativa dei microrganismi patogeni, inoculati artificialmente con concentrazioni decimali crescenti (10^2, 10^3, 10^6 UFC/g). Dall'analisi dei risultati ottenuti, è stata osservata la riduzione totale del carico microbico in un periodo di tempo che varia da poche ore a pochi giorni, a seconda del microrganismo considerato nella ricerca.

Parole chiave: Idrossido di calcio idrato, letame di pollame, riduzione microbica, *Salmonella* spp., *Escherichia coli, Enterococcus* spp.

How to cite
F. Carraturo, M.Morelli, M. Di Santo, P. Ambrosino, T. Crovella and M. Guida (2021). Survival of *Salmonella* Typhimurium, *Escherichia coli,* and *Enterococcus faecalis* in poultry manure samples, treated with different concentrations of hydrated calcium hydroxide. Bulletin of Regional Natural History (BORNH), Bollettino della Società dei Naturalisti in Napoli. Vol.1, n. 3, pp. 40 - 53 ISSN: 2724-4393.

Introduction

It is essential, in order to certify the quality and safety of fertilizers, to verify compliance with current regulations, which mainly require the microbiological safety of soil improvers. The legislation - EC Regulation 142/2011, Annex XI, Chapter I, Section 2, Point D - establishes a detection limit for *Escherichia coli* of 10^3 CFU/g, *Enterococci* of 10^3 CFU/g and the absence of *Salmonella* spp. in 25g of the product.
Poultry manure holds a significant agronomic value (Hutchison et al., 2004), in terms of the

contribution of organic matter and macro, meso- and micro-nutrients (Millner et al., 2014), and is therefore exploited and recovered in agriculture as an organic fertilizer, providing the nutrients subtracted from it to the soil with crops. The most common industrial treatment of poultry manure, useful for reducing humidity and transforming the product into a safe formulation at a sanitary level, involves drying the matrix by rapid heating at high temperature or slow heating at low temperature, to lower the humidity to a range between 10% and 15%. Poultry manure examined is provided by Agriges srl, a company located in the Benevento area that produces special fertilizers for organic and integrated agriculture, carrying out various treatments, including bactericidal approaches by heat treatments.

Poultry manure specimens employed in the present study, following the bactericidal treatment with hydrated calcium hydroxide, intended for use in agriculture, were stored at room temperature in aerobic conditions (Cotta et al., 2003).

The study pointed at assessing the efficacy of bacterial inactivation in poultry manure, using hydrated calcium hydroxide (Ruiz et al., 2008): experiments consisted on artificially spiking poultry manure with known concentrations of three pathogenic microorganisms, verifying how hydrated calcium hydroxide acts on the control of bacterial growth (Więckol-Ryk et al., 2020).

Different percentages of hydrated calcium hydroxide were tested, in relation to the proliferation or survival of *Escherichia coli* ATCC® 25922, *Salmonella* Typhimurium ATCC® 14028, and *Enterococcus faecalis* ATCC®19433 (Bennet et al., 2003; Park et al., 2003). The indicators employed in the present study (*Salmonella* spp., *Escherichia coli*, and *Enterococcus* spp.) play a crucial role in monitoring the health risks connected to fertilizers (Awasthi et al., 2018; Mohmed et al., 2013). Such pathogens furthermore represent the reference indicators for the treatment of residual biomass, such as low-risk animal by-products, and for the agronomic use of commercial organic fertilizer products derived from waste and organic waste. In parallel, the variations of pH and humidity in the samples were verified, since substantial variations of such parameters may support the decrease of the microbial growth (Heinonen-Tanski, et al., 2006; Andersson et al., 2001; Nyberg et al., 2011; Soliman et al., 2018), ensuring the safety and quality of the final fertilizing product.

Materials and Methods

Microbiological analysis of poultry manure

Before proceeding with the spiking of poultry manure with pathogenic microorganisms, samples were subjected to microbiological analyses to evaluate the presence of the pathogens in the matrix.

Microbiological analyses were carried out using standardized protocols: samples were weighed and homogenized, in order to obtain the desired solution. The sub-aliquots of the samples were homogenized in liquid and solid enrichment media, following the specific protocols to highlight the growth of each bacterium, allowing subsequent characterization and enumeration. The parameters were investigated at a temperature of 30-32 °C and aerobic conditions, close to the optimal temperature

range for the pathogens examined (ISO 16649:1:2018; ISO 6579:2008; ISO 7899:2:2003) (International Organization for Standardization 2018; International Organization for Standardization 2008; International Organization for Standardization 2003). Sample analysis was performed before the experimental protocol was started. *Salmonella spp*. was not detected, while fecal *Coliforms* and *Enterococci* were isolated at a concentration of about 10^5 CFU/g (Table 1) (International Organization for Standardization 2003; International Organization for Standardization 2004; International Organization for Standardization 2004; International Organization for Standardization 2008; International Organization for Standardization 2013; International Organization for Standardization 2018).

Bacterial inocula preparation

The strains used for artificial contamination of poultry manure were *Escherichia coli* ATCC® 25922, *Salmonella* Typhimurium ATCC® 14028, and *Enterococcus faecalis* ATCC®. The single inocula of the three strains were grown according to the metabolic needs of the microorganisms.
Inocula were kept under constant stirring at 200 rpm in 40 mL of Tryptic Soy Broth (TSB, OXOID) in sterile 50 mL tubes. To obtain a concentration of 10^8 cells/mL of each microorganism, the spectrophotometer was used, and an absorbance value at 560 nm wavelength was measured: a value of 0.125 O.D. indicates a reference concentration of 10^8 cells/mL. The inocula underwent ten-fold dilutions, obtaining the concentrations established for the experiment (10^6, 10^3 and 10^2 cells/mL).

Table 1. Results of the microbiological analyses carried out on the poultry manure samples (t_{PRE}), before proceeding with the artificial contamination using *E. coli, E. faecalis,* and *S.* Typhimurium, before treating the matrix with hydrated calcium hydroxide.

Parameter	Concentration [CFU/g]	Reference method
Total Bacterial Count at 37°C	$1{,}52*10^6$	UNI EN ISO 4833-1:2013
Enterococci	$3{,}25*10^5$	UNI EN ISO 21528-2:2004
Escherichia coli	$3{,}74*10^5$	UNI EN ISO 16649-2:2001
Clostridium perfringens	Absent	UNI EN ISO 7937:2004
***Salmonella* spp.**	Absent	UNI EN ISO 6579:2014
***Campylobacter* spp.**	Absent	UNI EN ISO 10272-1:2017

Contamination of poultry manure and bactericidal treatment

Aliquots of 100 g of poultry manure were weighed and poured into 9 sterile glass bottles with 500 mL each of sterile water. Samples were artificially spiked with serial ten-fold dilutions of the bacteria and incubated with a temperature range of 30-32 °C, in aerobic conditions. The experiment was conducted for 14 days. Before the artificial contamination (t_{PRE}), after the addition of hydrated calcium hydroxide (t_0) and after 1, 2, 7 and 14 days from contamination, the samples were subjected to qualitative and quantitative microbiological analyses in order to verify the concentrations of the three indicators in each sample. Analyses were conducted in triplicate.

The experimental design is following summarized: the manure was contaminated with different concentrations of *Escherichia coli* ATCC® 25922, *Salmonella* Typhimurium ATCC® 14028 and *Enterococcus faecalis* ATCC® 19433 (10^2, 10^3, 10^6 CFU/g). The samples were incubated at 30 °C under aerobic conditions for 16 hours and treated as follows:

- non-treated poultry manure spiked samples, considered as negative control (NC)
- poultry manure spiked samples treated with 15% hydrated calcium hydroxide
- poultry manure spiked samples treated with 25% hydrated calcium hydroxide
- microorganisms inocula in sterile water (to verify microbial growth without the contribution of poultry manure).

In addition, monitoring of temperature, pH and humidity were carried out. The experimental model is showed in detail in Figure 1.

As for Negative Control (NC) samples and bacterial inocula, in accordance with the company, we decided to inoculating microorganisms in sterile water, instead of supplemented growth media (e.g., Tryptone Soya Broth, OXOID), in order to verify the survival of the germs in absence of nutrients. The resulting curve refers to the decrease of microbial count in function of the lack of nutrients: the graph was compared to negative control and treated samples.

Results and Discussion

The synergistic effect of hydrated calcium hydroxide with the variation of pH and humidity were able to inhibit the growth and replication of the pathogens object of the study: therefore, a reduction in the microbial load of *Salmonella spp.*, *Escherichia coli* and *Enterococci faecalis* was observed.

Salmonella Typhimurium ATCC® 14028

The analysis of manure artificially spiked with 10^6 CFU/g of *Salmonella Typhimurium* is showed in Figure 2A. Analyzing the data of untreated manure, shortly after the artificial contamination, a 2-log decrease in the microbial load was observed. At day 1 (d_2), 72 hours after the artificial contamination, the resulting microbial load of the sample spiked with 10^6 CFU/g *Salmonella* Typhimurium is 10^3 CFU/g, steadily decreasing up to d_{14}. The outcomes may depend on an inhibiting action of the organic and inorganic components of the poultry manure on *Salmonella spp.* In poultry manure treated with 15% and with 25% hydrated calcium hydroxide, the microbial load die-off was evidenced, shortly after the application of hydrated calcium hydroxide.

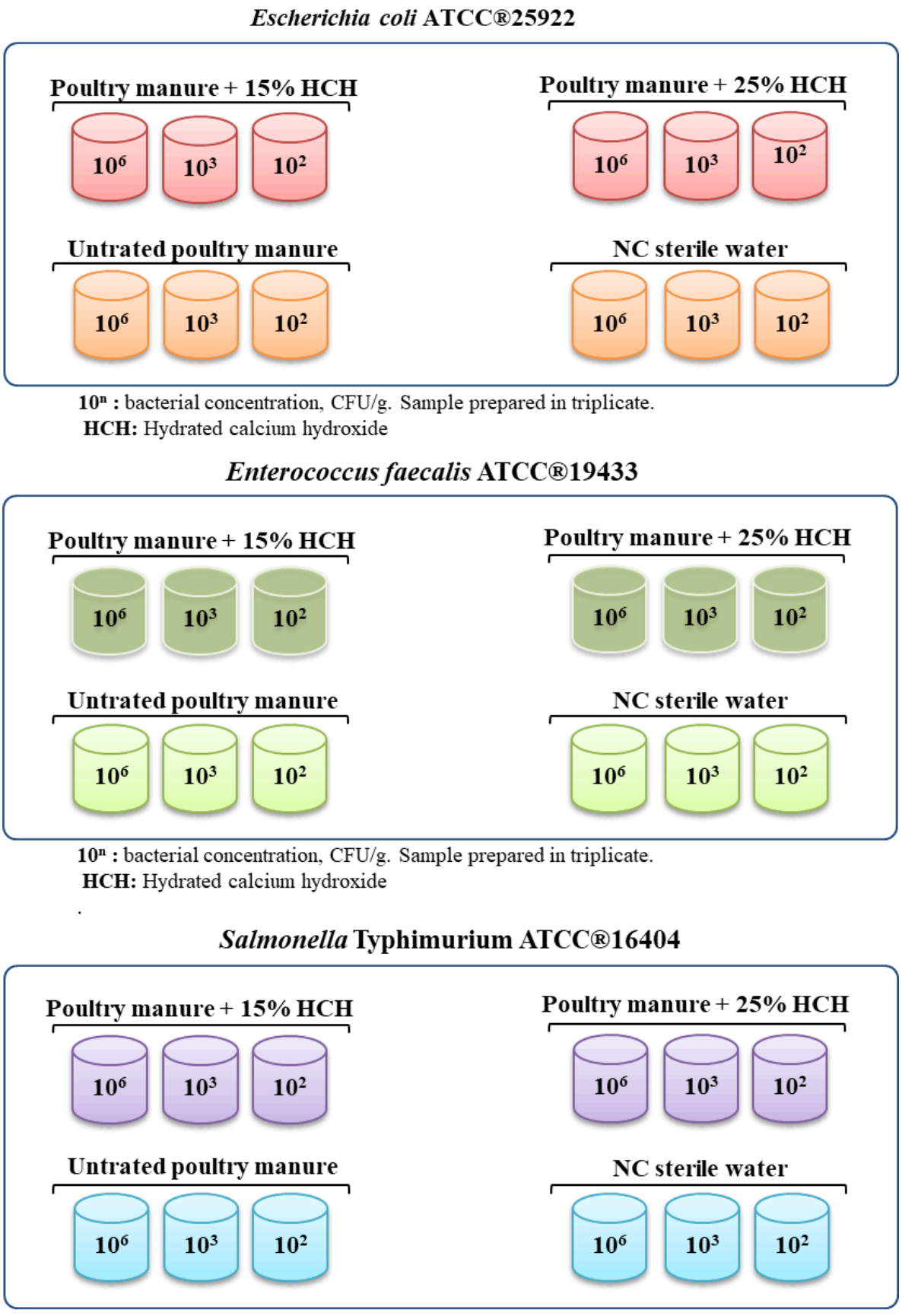

Figure 1: Experimental design of the samples analysed in the study.

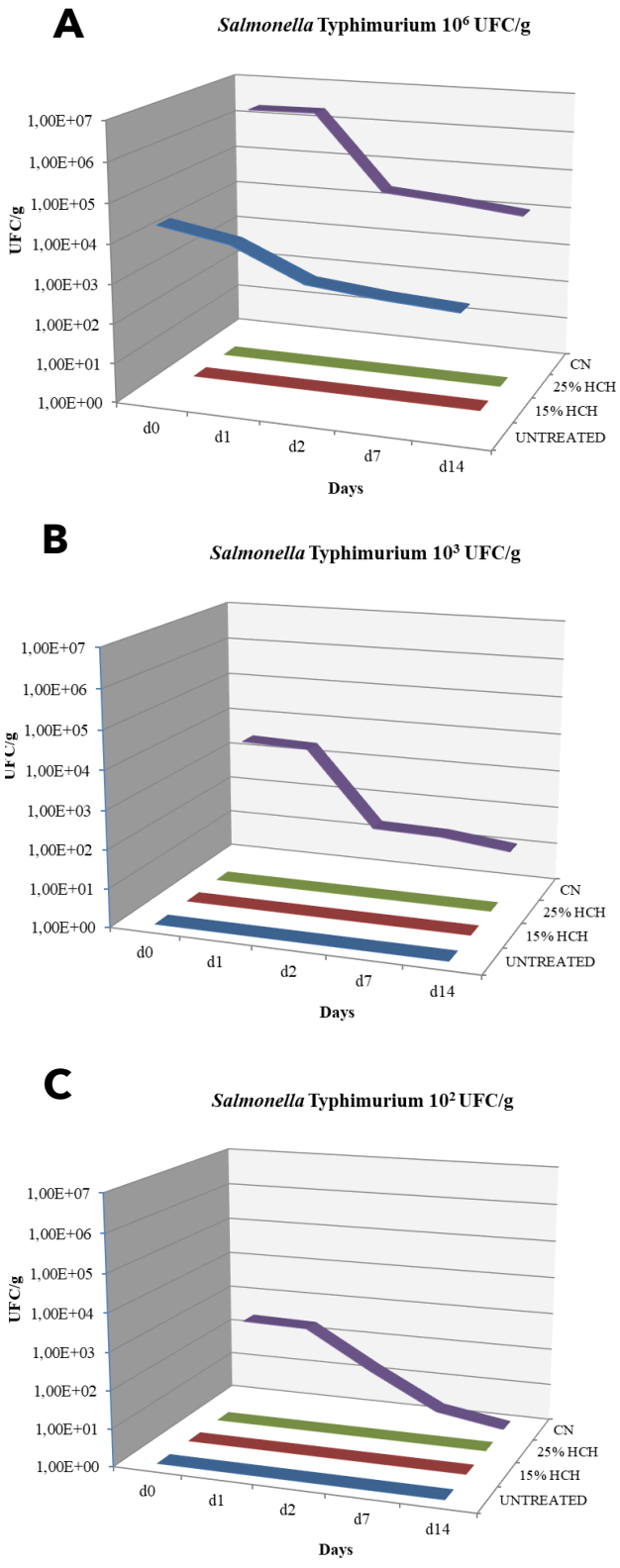

Figure 2: Trend of *Salmonella* Typhimurium in poultry manure starting from a concentration of A) 10^6 CFU/g B) 10^3 CFU/g.

In the negative control, not treated with hydrated calcium hydroxide, microbial loads gradually decrease, although less than in the untreated manure samples: in fact, a microbial load of about 10^4 CFU/g was observed between d_7 and d_{14}.

The analysis of poultry manure spiked with 10^3 CFU/g of *Salmonella Typhimurium* is described in Figure 2B. In the untreated poultry manure, a substantial decrease in the microbial load was observed since d_0. The manure treated with 15% and 25% hydrated calcium hydroxide showed the complete die-off of *Salmonella* spp. after the application of hydrated calcium hydroxide. In the negative control (NC) the load gradually decreases and a microbial load of about 10^2 CFU/g from d_2 was recorded.

The analysis of manure artificially contaminated with 10^2 CFU/g *Salmonella* Typhimurium is showed Figure 2C. Also in this case, the untreated manure, and the poultry manure treated with 15% and 25% hydrated calcium hydroxide, registered a huge decrease in the microbial load from day 0.In the negative control (NC), a microbial load < 10 CFU/g at d_1 was recorded.

Escherichia coli ATCC® 25922

The analysis of poultry manure artificially contaminated with 10^6 CFU/g *Escherichia coli* is showed in Figure 3A. In the untreated manure, the microbial load was doubled, as the artificially added concentration was added to that already present in the manure sample. A 1-log decrease in the microbial load was observed from d_2. At d_7 the resulting microbial load is 10^4 CFU/g, remaining constant until d_{14}. Poultry manure treated with 15% and 25% hydrated calcium hydroxide, showed a microbial die-off, after the application of hydrated calcium

hydroxide. In the negative control (NC) the microbial load remains constant, decreasing from d_{14}.

The analysis of manure contaminated with 10^3 UFC/g *Escherichia coli* is showed Figure 3B. Analyzing the untreated manure, that, nonetheless the previous presence of *E. coli* in the sample, a 1-log decrease in the microbial starting from d_2 is evident. At d_7 the resulting load is 500 CFU/g, constant until d_{14}.

The analysis of poultry manure spiked with 10^2 CFU/g *Escherichia coli* is showed in Figure 3C. Untreated manure microbial load decreased from d_2. At d_{14} the resulting charge is 50 CFU/g, constant until d_{14}. Poultry manure treated with 15% and 25% hydrated calcium hydroxide, showed a microbial load of 0 CFU/g after the application. Negative control (CN) loads decreased from d_7.

Enterococcus faecalis ATCC® 19433

In samples spiked with different concentrations of *Enterococcus faecalis,* a higher survival trend was evident, also considering that, at d_0, the microbial load added artificially was summed to that already present in the manure samples. Indeed, *Enterococcus faecalis* is the only microorganism in the study able to survive, although for not more than 48 hours, following the application of hydrated calcium hydroxide to the manure.

The analysis of manure spiked with 10^6 CFU/g *Enterococcus faecalis* is available in Figure 4A. As for untreated manure, the decrease in microbial load is evident already from d_2. At d_7 the resulting charge is 10^5 CFU/g. In manure treated with 15% and 25% hydrated calcium hydroxide, the microbial load

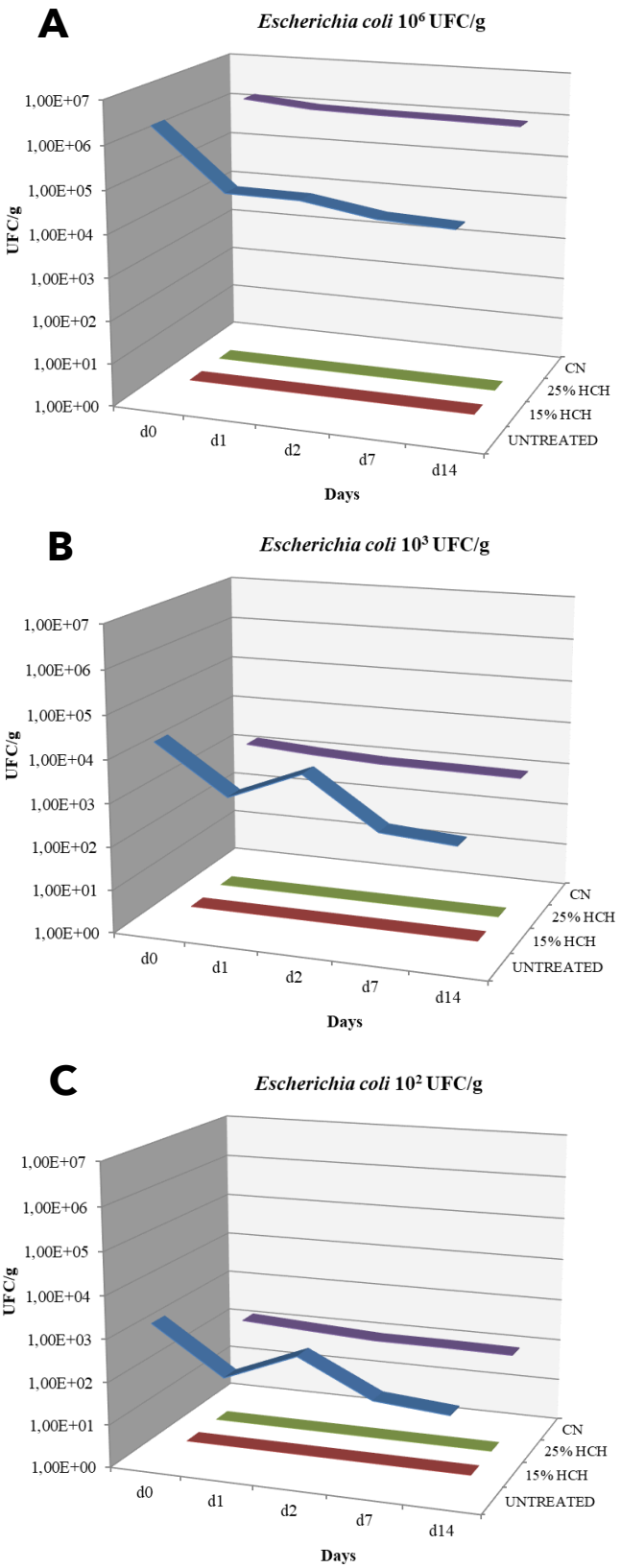

Figure 3: Trend of *Escherichia coli* in poultry manure starting from a concentration of A) 10^6 CFU/g B) 10^3 CFU/g and C) 10^2 CFU/g.

decreased significantly showing a complete

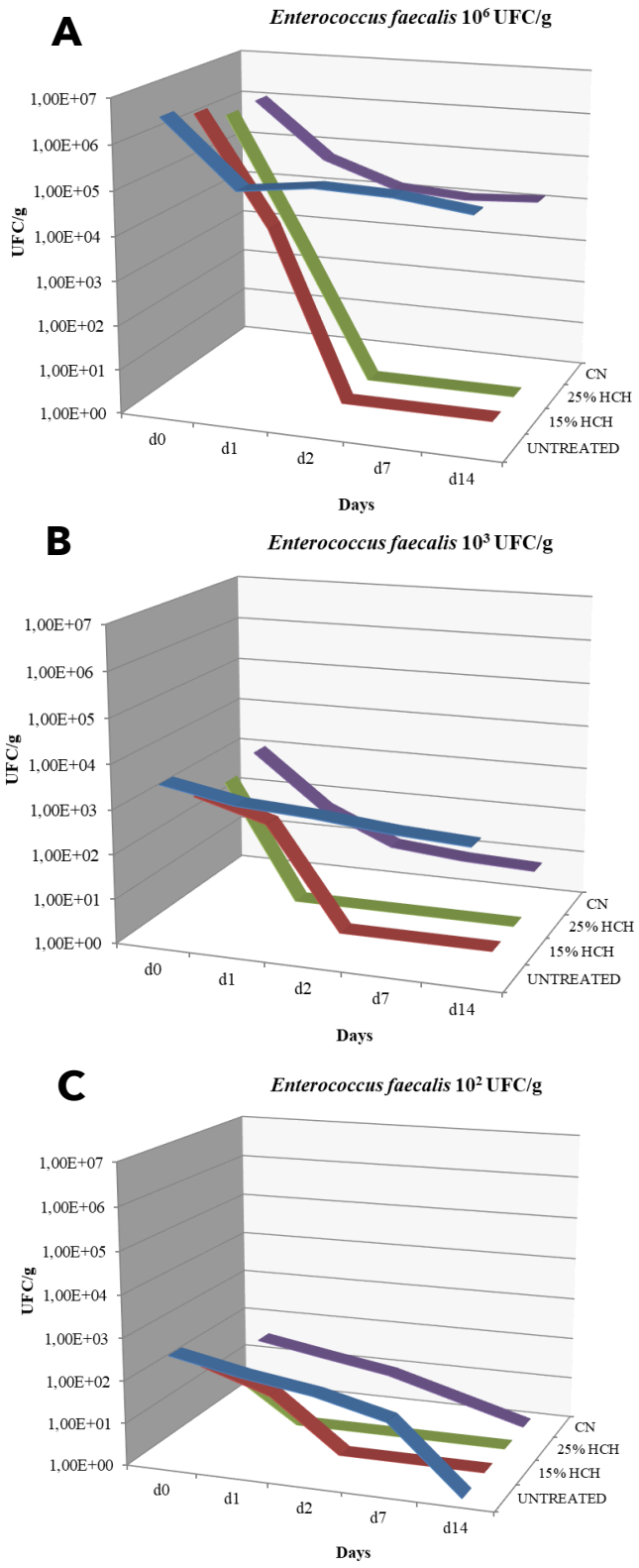

Figure 4: Trend of *Enterococcus faecalis* in poultry manure starting from a concentration of A) 10^6 CFU/g B) 10^3 CFU/g and C) 10^2 CFU/g.

die off at d_2. The microbial load of the negative control (NC) is constant and decreases from d_2.

The analysis of manure artificially contaminated with 10^3 CFU/g of *Enterococcus faecalis* is showed in the Figure 4B. The untreated manure showed a decrease in the microbial load from d_7. Microbial load of poultry manure treated with 15% and 25% hydrated calcium hydroxide, decreases significantly.

Analysis of manure spiked with 10^2 CFU/g *Enterococcus faecalis* is showed in Figure 4C. The untreated manure showed a starting microbial load < 400 CFU/g; load resulted reduced between d_7 and d_{14}. As for poultry manure treated with 15% and 25% hydrated calcium hydroxide, at d_1, microorganisms decrease significantly: the microbial die-off is obtained in 24 hours with 25%, and after 48 hours with 15% hydrated calcium hydroxide. In the negative control (NC) sample, microbial load decreases from d_2.

pH and Humidity

In parallel with microbiological analysis, the variations of pH and humidity in the samples of poultry manure treated with hydrated calcium hydroxide were measured (Soliman et al., 2018).

The graphs showed in Figures 5 and 6 describe the trend of the pH and humidity parameters registered in the samples spiked with the three pathogenic microorganisms and subjected to bactericidal treatment, employing different percentages of hydrated calcium hydroxide.

The pH values of artificially contaminated and non-treated poultry manure samples are between 8 and 10 over time,

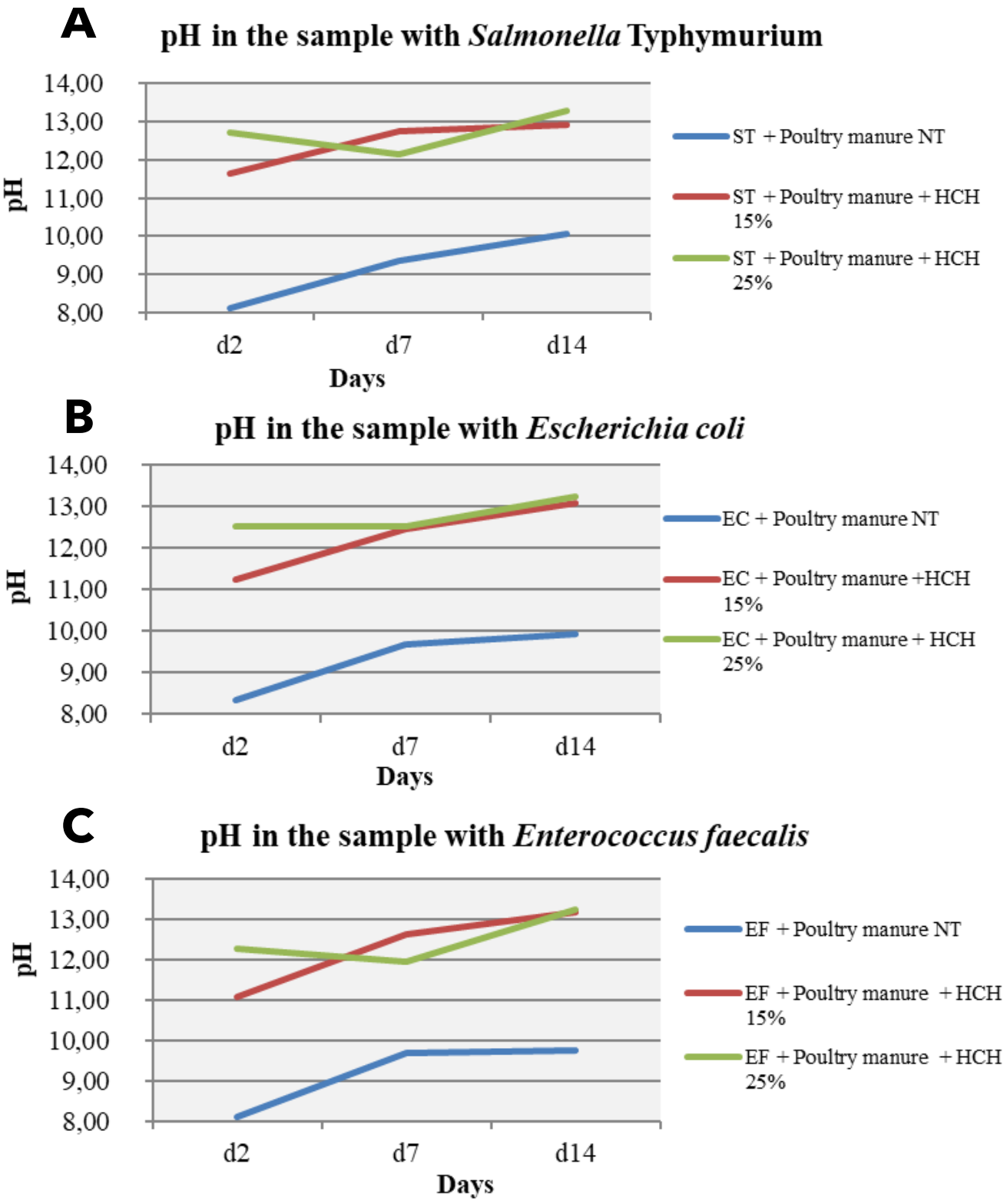

Figure 5: Trend of pH in poultry manure spiked with A) *Salmonella* Typhimurium [ST: *Salmonella* Typhimurium; NT: Not Treated; HCH: hydrated calcium hydroxide], B) *Escherichia coli* [EC: *Escherichia coli*; NT: Not Treated; HCH: hydrated calcium hydroxide] and C) *Enterococcus faecalis* [EF: *Enterococcus faecalis*; NT: Not Treated; HCH: hydrated calcium hydroxide].

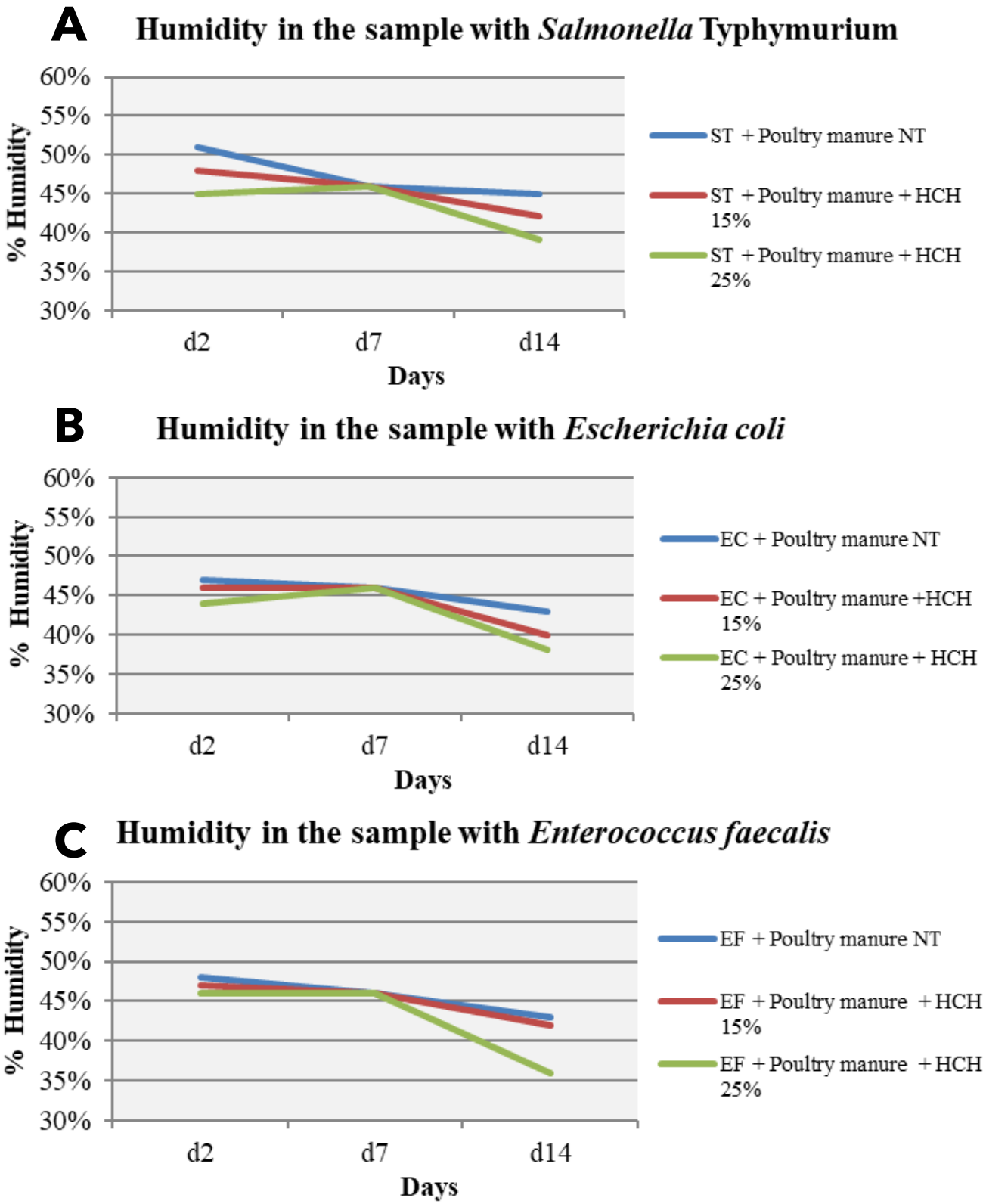

Figure 6: Trend of humidity in poultry manure spiked with A) *Salmonella* Typhimurium [ST: *Salmonella* Typhimurium; NT: Not Treated; HCH: hydrated calcium hydroxide], B) *Escherichia coli* [EC: *Escherichia coli*; NT: Not Treated; HCH: hydrated calcium hydroxide] and C) *Enterococcus faecalis* [EF: *Enterococcus faecalis*; NT: Not Treated; HCH: hydrated calcium hydroxide].

precisely between d_2, with a pH at 8, and d_{14}, with a pH at 9. The addition of 15% hydrated calcium hydroxide to manure samples, artificially contaminated with the three bacteria, causes an increase of the pH to values between 11 (d_2) and 13.1 (d_{14}). Evaluating the samples in which 25% hydrated calcium hydroxide was added, pH values between 12.5 (d_2) and 13.3 (d_{14}) were recorded.

With regards to humidity, in artificially contaminated samples treated with hydrated calcium hydroxide, a decrease from an average value of 49% (d_2), to 43.5% (d_{14}) was observed.

Conclusions

The components of the manure enhance the rapid elimination of the microbial load of *Salmonella* Typhimurium (Dai Pra et al., 2009), *Escherichia coli*, and *Enterococcus faecalis*.

The application of hydrated calcium hydroxide as a sanitizing and microbicidal agent, and the consequent variations in pH (alkaline) and humidity (which decreases) (Nyberg et al.,2011), confirm the low risk of survival of the indicator germs in the matrix under examination over 24 hours following the treatment. Analysing the obtained data, nevertheless the pH increase strongly inhibits the microbial proliferation and survival, it is essential to underline that pH higher than 8-9 may damage the manure, following the treatment. As a practical application, the exploitation of the treated poultry manure as a fertilizer could be performed by employing pH correctors, such as citric acid (also a food supplement), able to stabilize the pH at a 7-8 value.

Salmonella Typhimurium loads < 10^3 CFU/g are able to survive less time than higher loads (10^6 CFU/g), both in untreated and treated poultry manure: the condition contributes to reducing the microbiological risk from Salmonella spp. Escherichia coli and Enterococcus faecalis are able to resist for higher time at the experimental conditions, especially at high microbial loads (> 10^6 CFU/g). Nevertheless, the survival risk of Escherichia coli in the matrix is low, considering that the contaminating load is completely removed within 2 hours of treatment with hydrated calcium hydroxide. The survival in the treated matrix is higher for Enterococcus faecalis, which, especially at high concentrations (10^6 CFU/g), survives in the hours following the treatment: however, the microorganism is no longer detectable within the 24 hours following the treatment with hydrated calcium hydroxide.

Considering that the experimental protocol included microbial loads of pathogens and indicators far higher than those potentially detected in real environmental conditions, it is evident that the survival of the pathogens and indicators is strongly limited in the process of manure storage, following the treatment with hydrated calcium hydroxide at the 15% and 25% concentrations; consequently, the risk of survival of the microorganisms evaluated has to be considered low.

Author contributions

Conceptualisation, M. Guida, F. Carraturo, and M. Di Santo; data curation, F. Carraturo; formal analysis, F. Carraturo and M. Guida; investigation, F. Carraturo; methodology, F. Carraturo, P. Ambrosino and M. Morelli; project administration, M. Guida, F. Carraturo and M. Di Santo; resources, P. Ambrosino, M.

Di Santo and M. Guida; supervision, M. Guida and P. Ambrosino; validation, F. Carraturo and M. Morelli; visualization, P. Ambrosino and T. Crovella; writing–original draft preparation, F. Carraturo and M. Morelli; writing–review and editing, M. Guida and F. Carraturo.

References

Andersson S., Nilsson S. I. 2001. Influence of pH and temperature on microbial activity, substrate availability of soil–solution bacteria and leaching of dissolved organic carbon in a mor humus. Soil Biol Biochem 33, 1181–1191.doi: 10.1016/S0038-0717(01)00022-0.

Awasthi M. K., Chen H., Wang Q., Liu T., Duan Y., Awasthi S. K., Ren X., Tu Z., Li J., Zhao J., Zhang Z. 2018. Succession of bacteria diversity in the poultry manure composted mixed with clay: Studies upon its dynamics and associations with physico chemical and gaseous parameters Bioresour Technol. 2018 Nov;267:618-625. doi: 10.1016/j.biortech.2018.07.094.

Bean C. L., Hansen J., Margolin A. B., Balkin H., Batzer G., Widmer G. 2007. Class B Alkaline Stabilization to Achieve Pathogen Inactivation Int. J. Environ. Res. Public Health 2007, 4(1), 53-60. doi:10.3390/ijerph2007010009.

Bennet D. D., Higgins S. E., Moore R. W., Beltran R., Caldwell D. J., Byrd J. A., Hargis B. M. 2003. Effects of lime on *Salmonella enteritidis* survival in vitro. J Appl Poult Res 12, 65–68, doi: 10.1093/japr/12.1.65.

Boost M. V., Poon C. S. The effect of a modified method of lime-stabilisation sewage treatment on enteric pathogens. 1998. Environ. Int., 24 pp. 783-788 doi: 10.1016/S0160-4120(98)00067-1.

Cotta M. A., Whitehead T. R., Zeltwanger R. L. 2003. Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits Environ Microbiol 2003 Sep;5(9): 737-45. doi: 10.1046/j.1467-2920.2003.00467.x.

Dai Pra M. A., Kunde Corrêa É., Roll V. F., Xavier E. G., Lopes D. C. N., Fernandes Lourenço F., Zanusso J. T., Piccini Roll A. 2009. Uso de cal virgem para o controle de *Salmonella* spp. e *Clostridium* spp. em camas de aviário Cienc. Rural vol.39 no.4 doi:10.1590/S0103-84782009005000028

Heinonen-Tanski H., Mohaibes M., Karinen P., Koivunen, J. 2006. Methods to reduce pathogen microorganisms in manure. Livestock Science, 102(3), pp 248–255. doi: 10.1016/j.livsci.2006.03.024.

Hutchison M. L., Walter L. D., Moore A., Crookes K. M., Avery S. M., 2004. Effect of length of time before incorporation on survival of pathogenic bacteria present in livestock wastes applied to agricultural soil. Appl. Environ. Microbiol. 70, pp 5111–5118. doi: 10.1128/aem.70.9.5111-5118.2004.

Nyberg K. A., Vinnerås B., Lewerin S.S, Kjellberg E., Albihn A. 2011. Treatment with Ca(OH)2 for inactivation of *Salmonella* Typhimurium and *Enterococcus faecalis* in soil contaminated with infected horse manure J Appl Microbiol. 2011 Jun; 110(6):1515-23 doi:10.1111/j.1365-2672.2011.05006.x.

International Organization for Standardization (ISO). 2003. ISO 7899:2:2003: Water quality - Research and enumeration of intestinal enterococci - Membrane filtration method. Available to: http://store.uni.com/catalogo/uni-en-iso-7899-2-2003.

International Organization for Standardization (ISO). 2004. ISO 21528-2:2004: Microbiology of food and animal feeding stuffs -- Horizontal methods for the detection and enumeration of Enterobacteriaceae Colony-count method. Available to: http://store.uni.com/catalogo/iso-21528-2-2004.

International Organization for Standardization (ISO). 2004. ISO 7937:2004: Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of *Clostridium perfringens* - Colony-count technique. Available to: http://store.uni.com/catalogo/iso-7937-2004.

International Organization for Standardization (ISO). 2008. ISO 6579:2008, Microbiology of food and animal feed - Horizontal method for the detection of *Salmonella* spp. Available to: http://store.uni.com/catalogo/uni-en-iso-6579-2008.

International Organization for Standardization (ISO). 2013. UNI EN ISO 4833-1:2013: Microbiology of the food chain - Horizontal method for the enumeration of microorganisms - Part 1: Colony count at 30 degrees C by the pour plate technique. Available: http://store.uni.com/catalogo/uni-en-iso-4833-1-2013.

International Organization for Standardization (ISO). 2018. ISO 16649-1:2018: Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* – Part 1: Colony-count technique at 44 degrees C using membranes and 5-bromo-4-chloro-3-indolyl beta-D-glucuronide. Available to: https://www.iso.org/standard/64951.html.

Park, G.W., Diez-Gonzalez, F. 2003. Utilization of carbonate and ammonia-based treatments to eliminate *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104 from cattle manure. J Appl Microbiol 2003;94(4):675-85. doi: 10.1046/j.1365-2672.2003.01899.x.

Ruiz V., Ruiz D., Gernat A.G., Anderson K., Wineland M., Grimes J. 2008. The Effect of Quicklime (CaO) on Litter Condition and Broiler Performance Pollame Sci., 87, pp. 823 – 827. doi: 10.14202/vetworld.2018.1064-1073.

Soliman E. S, Sallam N. H, Abouelhassan E.M. 2018. Effectiveness of poultry litter amendments on bacterial survival and *Eimeria* oocyst sporulation. Vet World. 2018 Aug;11(8):1064-1073. doi: 10.14202/vetworld.2018.1064-1073.

Więckol-Ryk A., Białecka B., Maciej T. 2020. Effect of Green Oxidizing Agent on Inhibition of *Escherichia coli*. Present in Livestock Wastes. Water Air Soil Pollut (2020) 231: 466. doi: 10.1007/s11270-020-04824-3.

Bulletin of Regional Natural History (BORNH)
ISSN 2724-4393.