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ORIGINAL ARTICLE

Viability of zoonotic pathogens *Escherichia coli* and *Salmonella* in swine manure slurries with and without a urease inhibitor and thymol

J.E. Wells and V.H. Varel

USDA-ARS, U.S. Meat Animal Research Center, Clay Center, NE, USA

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E. coli O157:H7, manure, *Salmonella enterica*, swine, *Typhimurium,* urea.

Correspondence
James E. Wells, U.S. Meat Animal Research Center, PO Box 166, Clay Center, NE 68933-0166, USA.
E-mail: jim.wells@ars.usda.gov

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Abstract

Aims: To determine the effects of urine, a urease inhibitor and/or an odour-reducing antimicrobial compound, on zoonotic pathogens in swine manure slurries.

Methods and Results: Swine faeces were collected and blended with different amounts of urine. Marker strains of *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Escherichia coli* O157:H7 were inoculated into the manure slurries with or without the urease inhibitor, *N*-(*n*-butyl) thiophosphoric triamide, or the antimicrobial compound thymol. In slurries containing approximately 1 : 1 or 2 : 1 of urine and faeces, the decreases in colony forming units (CFU) of *Salm.* Typhimurium and *E. coli* were similar and few counts were observed after 14 days. When the urine content of the slurry was increased to 5 : 1, both strains died off rapidly. When NBPT was added to the slurries, pathogen CFU was not affected in the 1 : 1 slurry. The 2 : 1 and 5 : 1 slurries had higher urea content and NBPT increased CFU of both pathogens. Thymol addition rapidly killed the pathogens and few CFU of *Salmonella* or *E. coli* were observed after day 1.

Conclusions: These experiments demonstrate that urea hydrolysis in swine manure affects pathogens. Inhibition of urea hydrolysis may promote pathogen viability.

Significance and Impact of the Study: Manure amendments that prevent urea hydrolysis may promote pathogen persistence. Additional treatments with antimicrobials may be required to kill pathogens.

Introduction

Animals commonly excrete zoonotic pathogens in the faeces and pathogen persistence in animal waste is a concern. Manure from animal waste in many animal production facilities are stored and later applied to land as fertilizer or soil conditioner. In the United States, manure application to land is often determined by the nitrogen or phosphorus content. Most of the nitrogen is excreted into the waste via the urine as urea, but urea is rapidly hydrolysed in manure to ammonia. The ammonia can volatilize, thus increasing ammonia emissions and decreasing the fertilizer value of the manure.

Swine production facilities utilize a variety of systems to manage the animal wastes. Pit storage systems under swine pens are common in the central and northern United States. In manures from different swine production systems, a variety of bacterial pathogens, including *Escherichia coli* O157, *Salmonella*, *Campylobacter*, *Listeria* and *Yersinia* have been detected (Hutchison *et al.* 2004, 2005; Bhaduri *et al.* 2005). Urea hydrolysis in manure slurries has been shown to decrease pathogens in dairy cattle manure slurries (Diez-Gonzalez *et al.* 2000), but little is known about the role of urea hydrolysis in swine manure slurries.

Treatments such as urease inhibitors may improve the nitrogen content and increase the value of the manure

while decreasing ammonia emissions (Varel 1997; Varel *et al.* 1999). However, inhibition of urea hydrolysis may impact pathogen persistence and increase the potential for pathogen transmission to the environment. If urea hydrolysis affects pathogen survival in swine manures, then additional treatments to kill pathogens may be warranted if urease inhibitors are used. The purpose of this study was to examine the effects of urea levels on pathogen persistence and determine if inhibition of urea hydrolysis allowed pathogens to persist in the manure slurry. Additional treatments with thymol, an antimicrobial plant essential oil effective at controlling manure odours (Varel and Miller 2001), were performed to determine if pathogen persistence was eliminated.

Materials and methods

Chemicals

Chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA), with the exception of *N*-(*n*-butyl) thiophosphoric triamide (NBPT, in commercial preparation Agrotain, Agrotain International, St Louis, MO, USA).

Slurries of swine waste preparation

Swine slurries were processed similar to previous studies (Varel and Miller 2000, 2004). Faeces were collected (within 12 h of excretion) and pooled as a composite for this study from pen floors of six pens (2 kg faeces each pen) of swine (10 animals per pen, 20–24 weeks of age) fed USMARC finishing diet (85% corn and 15% soyabean meal, see Yen *et al.* 2004 for complete composition). Pens had been cleaned of waste the previous evening. Urine was collected earlier from bladder catherized animals fed USMARC finishing ration. The urine was acidified with 6 N HCl (1 ml to each 1 l of urine) to preserve urea, pooled and stored frozen. The urease inhibitor, NBPT was mixed with the urine prior to slurry blending to yield 80 ppm final concentration in the manure slurries with NBPT (Varel 1997; Varel *et al.* 1999). Urine and faeces were mixed to form the different 650 ml manure slurry mixtures of approximately 1 : 1, 2 : 1 and 5 : 1, and blended for 1 min (Waring Inc., New Hartford, CT, USA). Thymol treatments were added to yield final concentration of 2000 ppm (Varel and Miller 2001, 2004) in the manure slurry and blended again. The 650 ml manure slurry was poured into 1500 ml jar. A subsample (50 g) of each slurry preparation prior to inoculation with pathogens was transferred to a glass tube and loosely capped for monitoring of coliform CFU over time. Each treatment (no addition, NBPT, thymol, and NBPT with thymol) was performed in triplicate jars at room temperature (19–20°C).

Bacterial strains and bacterial analyses

Cultures of *Escherichia coli* O157:H7 ATCC 43895 (streptomycin-resistant) or *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC14028 (nalidixic acid-resistant) were grown overnight (18 h) in Difco tryptic soy broth (Becton Dickinson Co., Sparks, MD) at 37°C and allowed to set at room temperature for 2 days. Previous work with faeces has demonstrated enhanced viability over time with these procedures (Wells *et al.* 2005). The slurries were inoculated with both *E. coli* O157:H7 streptomycin-resistant and *Salm.* Typhimurium nalidixic acid-resistant strains, each at a concentration of approximately 1×10^6 bacteria g^{-1} slurry. To determine the pathogen numbers, slurries were sampled over several days until pathogens were no longer countable. Each jar of manure slurry was mixed well and a 1 g sample was collected. Each sample was diluted into 9 ml of tryptic soy broth (Difco) and vortexed vigorously for 1 min. Samples were diluted further in 10-fold increments to 10^{-3} and 100 μl of each dilution was plated in duplicate onto MacConkey-Sorbitol agar supplemented with streptomycin (250 μg ml^{-1} final) to enumerate the inoculated *E. coli* O157:H7 and tryptic soy agar supplemented with nalidixic acid (250 μg ml^{-1} final) to count the inoculated *Salm.* Typhimurium. Agar plates were incubated overnight at 37°C and colonies were counted. Microbial counts (CFU g^{-1} faeces) were determined from the faecal dilutions, plated volume and counted colonies (streptomycin-resistant or nalidixic acid-resistant) for each plate. Based on duplicate plates with 100 μl of 10^{-1} dilution (our lowest dilution), the limit of detection was 5×10^1 CFU of each inoculated bacteria per gram faeces. When the inoculated bacterial population decreased below these detectable limits, we continued sampling for three additional days and for confirmation. Bacterial colonies (two–three colonies from each agar plate with colonies) were tested and to confirm bacterial pathogen using agglutination tests kits, anti-*E. coli* O157 Latex Test (Oxoid Ltd, Basingstoke, UK) and anti-*Salmonella* Latex Test (Oxoid). All tested colonies reacted positively with the respective latex. No colonies with antimicrobial resistance to streptomycin or nalidixic acid were noted at the lowest dilution of samples from slurries not inoculated (subsamples) with our bacterial test strains on day 0 or day 14.

Coliform bacteria were enumerated using Petrifilms (3M Microbiology Products, St Paul, MN, USA). Slurry samples (1 g each) were diluted into tryptic soy broth (Difco) as above and 1 ml of each respective dilution was transferred to the film. Petrifilms were incubated

overnight at 37°C and gas-producing colonies (red and blue) were enumerated for coliform CFU.

Analytical methods

Manure slurry pH was determined in the jars using a combination pH electrode and a PHM 83 pH meter (Radiometer America, Cleveland, OH, USA). Samples for analytical analysis were taken on days 0, 1, 4, 7 and 14. A 15ml slurry sample was collected from each jar after brief swirling of contents. An equal volume of 0·5 mol l^{-1} H_2SO_4 was added to each sample and mixed. The acidified sample was centrifuged at 2000 ***g*** (20 min, 4°C) and the supernatant was decanted and stored at −20°C until analysed for urea (Varel and Miller 2000). Urea content was determined using a modification of the Sigma Blood Urea Nitrogen kit (BUN; Sigma) procedure. Briefly, samples were diluted 10-fold and 20 μl was added to 300 μl BUN acid and 200 μl BUN color in a glass test tube. Samples with reagents were boiled for 10 min and immediately transferred to cold-water bath for 5 min. A 300 μl aliquot of each sample was transferred to a 96well microtitre plate. Absorbance at 515 nm was read using a Bio-Tek Ceres UV900C microplate reader (Bio-Tek, Winooski, VT, USA) and urea content was determined from linear regression to a standard curve.

Statistical analysis

Microbial numbers (CFU) were transformed to log base 10 ($\log_{10}$ CFUs g^{-1} faeces) and plotted over time. The counts over time were subjected to linear regression analysis (Kaleidagraph 3.6.4; Synergy Software, Reading, PA, USA) to determine the rate of change in CFU in each slurry mix. Rates, CFU counts and analytical results (urea and pH) for the three types of slurries and the four treatments were analysed as a 3×4 factoral design using GLM procedure in SAS Version 6.12 (SAS Institute Inc., Cary, NC, USA, 1996) for significant differences ($P < 0{\cdot}05$). The experimental unit was sample jar and the statistical model included the effects of slurry type, slurry treatment, time (except for rate analysis), and their interactions. Means for triplicate sample jars are reported ($n = 3$).

Results

The initial urea concentration increased by more than twofold as more urine was added in the manure slurries, but little urea remained in any of the control (untreated) slurries by day 1 (Table 1). Addition of NBPT did not inhibit urea hydrolysis completely and some urea loss was observed in all slurry ratio mixes. In the 5 : 1 slurries,

Table 1 Effect of urease inhibitor (NBPT) and antimicrobial agent (thymol) in manure slurries with different ratios of urine to faeces on the residual urea concentration

		Urea (g l^{-1}) in manure slurries			
Slurry (urine : faeces)	Day	Control	NBPT	Thymol	NBPT and Thymol
1 : 1	0	3·4	3·4	3·4	3·4
	1	0·1^a	2·5^b	0·1^a	3·2^c
	4	0·1^a	1·1^b	0·1^a	2·3^c
	7	0·1	0·1	0·1	0·1
2 : 1	0	5·5	5·5	5·5	5·5
	1	0·1^a	4·9^b	0·1^a	5·2^c
	4	0·1^a	3·1^b	0·1^a	4·0^c
	7	0·1^a	0·8^b	0·1^a	1·9^c
5 : 1	0	8·2	8·2	8·2	8·2
	1	0·1^a	8·2^b	4·4^c	8·2^b
	4	0·1^a	8·0^b	1·1^c	8·0^b
	7	0·1^a	6·8^b	0·7^c	6·8^b
	14	0·1^a	5·2^b	0·2^a	6·1^c

Means are significantly different within row if superscript letters are different. Means are based on average of three observations per cell. The SEM based on GLM analysis was 0·065.

NBPT did sustain urea concentration for over 4 days, but by day 7 the urea concentrations started to slowly decrease. Thymol alone did not preserve the urea, but in combination with the NBPT, urea loss was slower than with NBPT alone ($P < 0{\cdot}05$). Slurry pH increased rapidly in the control slurries and was highest in the 5 : 1 control slurries (Table 2). Treatments with NBPT (alone or in combination with thymol) resulted in lower pH values ($P < 0{\cdot}05$) for most of the slurries measured until urea was hydrolysed. Treatments with thymol alone typically resulted in pH measurements equal to or greater than the controls ($P < 0{\cdot}05$). Indigenous coliform counts or CFU in subsamples decreased fastest (rate) in the 5 : 1 manure slurry (Table 3) and NBPT significantly minimized the decreases in CFU for the 2 : 1 and the 5 : 1 slurries ($P < 0{\cdot}01$).

In mixed slurries containing approximately 1 : 1 or 2 : 1 of urine and faeces, the decreases in CFU of the inoculated *E. coli* O157:H7 (streptomycin-resistant CFU) in untreated slurries were similar (rates are 0·282 and 0·291 $-\log_{10}$ CFU g^{-1} slurry day^{-1}, respectively, Table 4) and few numbers were detectable after 14 days (Fig. 1). In slurries with 5 : 1 urine to faeces, the rate in CFU reduction over time (rate) was more than fivefold greater ($P < 0{\cdot}001$) and no *E. coli* O157:H7 counts were observed after 3 days. Addition of NBPT to slurries did not significantly affect the rate of *E. coli* O157:H7 reduction with 1 : 1 slurries (Table 4), but did slow the rate in the 2 : 1 slurries by 15% (0·291 *vs* 0·246 $-\log_{10}$ CFU g^{-1} slurry day^{-1}, $P < 0{\cdot}01$) and 5 : 1 slurries by 67% (1·58 *vs* 0·527

Table 2 Effect of urease inhibitor (NBPT) and antimicrobial agent (thymol) in manure slurries with different ratios of urine to faeces on the slurry pH

Slurry (urine : faeces)	Day	pH of slurry			
		Control	NBPT	Thymol	NBPT and Thymol
1 : 1	0	7·07[a]	7·01[a]	7·85[b]	7·14[a]
	1	7·47[a]	6·66[b]	8·05[c]	6·86[b]
	4	7·52[a]	7·12[b]	7·55[a]	7·15[b]
	7	7·61[a]	7·38[b]	7·55[a]	7·38[b]
2 : 1	0	6·96[a]	6·75[b]	7·23[c]	6·80[a,b]
	1	8·53[a]	6·59[b]	8·75[c]	6·55[b]
	4	7·85[a]	7·50[b]	8·76[c]	7·51[b,d]
	7	7·96[a]	8·37[b]	8·68[c]	8·47[b,d]
5 : 1	0	6·76[a]	6·68[a]	6·99[b]	6·78[a]
	1	8·98[a]	6·36[b]	8·94[a]	6·58[c]
	4	8·85[a]	6·65[b]	9·03[a]	6·90[c]
	7	8·82[a]	6·86[b]	9·15[c]	8·09[d]
	14	9·15[a]	8·78[b]	9·16[a]	8·75[b]

Means are significantly different within row if superscript letters are different. Means are based on average of three observations per cell. The SEM based on GLM analysis was 0·062.

Table 3 The effects of NBPT and/or thymol on indigenous coliform counts in finishing swine manure slurries with different ratios of urine and faeces

Slurry (urine : faeces)	Loss in culturable coliforms (death rate, $\log_{10}$ CFU reductions g^{-1} slurry day^{-1})			
	Control	NBPT	Thymol	NBPT and Thymol
1 : 1	0·155[a]	0·155[a]	4·80[b]	4·87[b]
2 : 1	0·360[a]	0·206[b]	2·51[c]	5·02[d]
5 : 1	2·09[a]	0·325[b]	4·10[c]	4·17[c]

Means are significantly different within row if superscript letters are different. Means are based on average of three observations per cell. SEM = 0·02.

Table 4 The effects of NBPT and/or thymol on *Escherichia coli* O157:H7 counts in finishing swine manure slurries with different ratios of urine and faeces

Slurry (urine : faeces)	Loss in culturable *Escherichia coli* O157:H7 (death rate, $\log_{10}$ CFU reductions g^{-1} slurry day^{-1})			
	Control	NBPT	Thymol	NBPT and Thymol
1 : 1	0·282[a]	0·252[a]	4·46[b]	2·34[c]
2 : 1	0·291[a]	0·246[b]	4·65[c]	4·65[c]
5 : 1	1·58[a]	0·527[b]	4·68[d]	4·32[c]

Means are significantly different within row if superscript letters are different. Means are based on average of three observations per cell. SEM = 0·10.

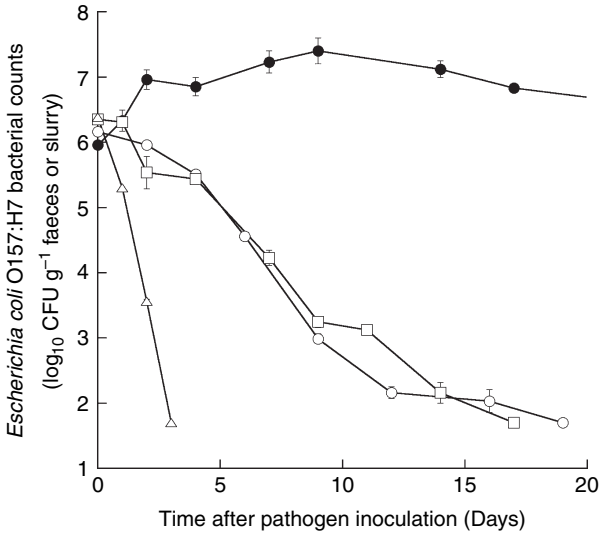

Figure 1 Counts ($\log_{10}$ g^{-1} sample) of *Escherichia coli* O157:H7 streptomycin-resistant CFU over time following inoculation into faeces (no urine, -●-) and manure slurries with different urine to faeces content (1 : 1, -○-; 2 : 1, -□-; and 5 : 1, -△-). Each point represents an average of three observations. Error bars represent SEM of each time point.

$-\log_{10}$ CFU g^{-1} slurry day^{-1}, $P < 0{\cdot}001$). Addition of thymol resulted in the fastest loss of CFU over time for all treatments ($P < 0{\cdot}001$) and no counts were observed after 2 days. The combination of thymol and NBPT in the 5 : 1 slurries yielded slower losses in CFU than the thymol alone ($P < 0{\cdot}001$).

In mixed slurries containing approximately 1 : 1 or 2 : 1 of urine and faeces, the decreases in CFU of *Salm.* Typhimurium (nalidixic acid-resistant CFU) in untreated slurries were similar (0·363 and 0·349 – $\log_{10}$ CFU g^{-1} slurry day^{-1}, Table 5), and few *Salm.* Typhimurium counts were detectable after 12 days. In slurries with 5 : 1 of urine to faeces, the rate of CFU loss was nearly sixfold greater ($P < 0{\cdot}001$) and no counts were observed after 2 days. Addition of NBPT to slurries did not significantly affect the rate of decreases in viable CFU with 1 : 1 or 2 : 1 slurries (Table 5), but did slow decreases in CFU in the 5 : 1 slurries by 75% (2·08 *vs* 0·515 $-\log_{10}$ CFU g^{-1} slurry day^{-1}, $P < 0{\cdot}001$). Addition of thymol resulted in rapid loss of CFU for all treatments ($P < 0{\cdot}001$) and no viable counts were observed after 2 days.

Discussion

Most of the nitrogen in animal waste comes from urea excreted in the urine, but the urea is rapidly hydrolysed to ammonia and subsequently volatilized into the air.

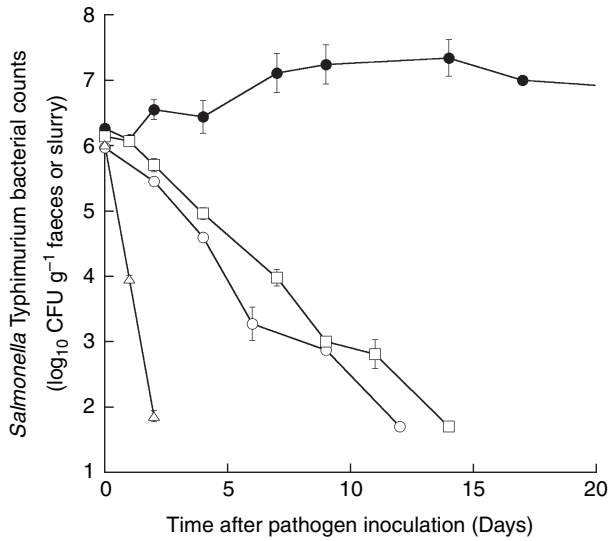

Figure 2 Counts ($\log_{10}$ g^{-1} sample) of *Salmonella* Typhimurium nalidixic acid-resistant CFU over time following inoculation into faeces (no urine, -●-) and manure slurries with different urine to faeces content (1 : 1, -○-; 2 : 1, -□-; and 5 : 1, -△-). Each point represents an average of three observations. Error bars represent SEM of each time point.

Table 5 The effects of NBPT and/or thymol on *Salmonella* Typhimurium viability in finishing swine manure slurries with different ratios of urine and faeces

	Loss in culturable *Salmonella* Typhimurium (death rate, $\log_{10}$ CFU reductions g^{-1} slurry day^{-1})			
Slurry (urine : faeces)	Control	NBPT	Thymol	NBPT and Thymol
1 : 1	0·363[a]	0·406[b]	4·27[c]	2·14[d]
2 : 1	0·349[a]	0·343[a]	4·94[b]	4·94[b]
5 : 1	2·08[a]	0·515[b]	4·31[c]	4·31[c]

Means are significantly different within row if superscript letters are different. Means are based on average of three observations per cell. SEM = 0·01.

Preserving urea can increase the nitrogen retention in the manure and minimize ammonia emissions. Previous work has demonstrated that treatments with urease inhibitors can increase nitrogen content on feedlot surfaces (Varel *et al.* 1999; Shi *et al.* 2001). However, urea hydrolysis has been shown to promote the death of pathogens as a result of the products of urea hydrolysis (Diez-Gonzalez *et al.* 2000; Park and Diez-Gonzalez 2003). As a consequence, inhibition of urea hydrolysis could prolong pathogen viability.

Manure composition may impact pathogen persistence, yet little information or consideration has been reported on the impact of urine or urea components on these bacteria in swine manure. In published standards (ASAE 1993), reported data suggest that urine and faeces are produced in near equal amounts by swine. In the manure slurries prepared for this study with 1 : 1 and 2 : 1 urine to faeces ratios, the addition of urease inhibitor was not effective at preserving urea. In these studies, the urea content in the slurry was low and less than would be expected based on metabolic trials with finishing swine. In previous metabolic work with swine in which the urine was collected, the urine to faeces ratios were 5 : 1 or greater (Yen *et al.* 2004) and thus our work with lower ratios in slurries was not representative of the growing animal's urea excretions. Based on this previous metabolic trial, the urine to faeces ratio was increased to 5 : 1 in a set of slurries. In the 5 : 1 slurry studies, the urea concentration was highest but still disappeared rapidly in the untreated samples. Additions of NBPT did inhibit the loss of urea in the 5 : 1 slurries for several days, and this may be a result of lower faecal matter and lower amounts of bacterial ureases in these slurries.

Pathogen decrease was fastest when the urea concentration in the slurries was the highest. Previous work by Diez-Gonzalez *et al.* (2000) demonstrated in dairy cattle manure that urine to faeces ratios greater than 2·2 resulted in the rapid death of non-pathogenic *Escherichia coli*. In the experiments of Diez-Gonzalez *et al.* (2000), bacterial viability decreased significantly with alkaline pH (pH > 8·5) and could be mimicked with sodium carbonate and sodium hydroxide in place of urinary urea. Subsequent work has reaffirmed the potential for urea to kill pathogens in dairy cattle waste and implicated ammonia to play a role in addition to carbonate anion (Park and Diez-Gonzalez 2003). In our untreated slurry experiments, the initial pH of the 5 : 1 urine to faeces slurries was 6·8 and increased rapidly to pH of 9·0 after 1 day, whereas the 1 : 1 urine to faeces slurries changed little over the first 7 days (pH < 7·7). Inhibition of urease activity with NBPT sustained lower slurry pH and prolonged observed pathogen counts. In addition, pathogens persisted for weeks in studies with swine faeces and no urine (Figs 1 and 2). Together, these results not only reaffirm the role that urea hydrolysis may play with pathogen persistence in swine manure slurries, but demonstrate the need to carefully consider manure management strategies that only preserve urea.

Thymol was effective at killing the target pathogens, and this treatment eliminated pathogens when slurries were also treated with NBPT to preserve the urea. Previous work has demonstrated that thymol can inhibit the production of odour compounds and also eliminate coliforms and generic *E. coli* populations (Varel and Miller 2004). Much of the previous work with essential oils has been with pure cultures of specific pathogens (Burt 2004). This study demonstrates the ability of thymol to kill

specific zoonotic pathogens in a complex manure environment, including the problematic pathogen in swine production facilities, *Salm.* Typhimurium.

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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