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Abstract. An incomplete anoxic fermentation of livestock waste results in offensive odor emissions. Antimicrobial additives may be useful in controlling odor emissions and pathogens. Natural antimicrobial compounds, carvacrol or thymol at 16.75 mM (2.5 g/l) completely inhibited the production of the offensive odor compounds, isobutyrate, valerate, isovalerate, and cresol, and significantly reduced other short-chain volatile fatty acids and gas emissions from swine waste. Fecal coliforms were reduced from 6.3×10^6 to 1.0×10^3 cells per ml 2 days after treatment with carvacrol (13.3 mM) and were not detectable within 14 days. Total culturable anaerobic bacteria were reduced from 12.4×10^{10} to 7.2×10^8 cells per ml after 2 days and were suppressed below this level for 28 days. Lactate production was not prevalent in untreated swine waste indicating that the microbial populations differ from those in cattle waste. Carvacrol and thymol were stable in swine waste under anoxic conditions for 62 days with 90 to 95% of the additive being recovered in the waste solids. In conclusion, carvacrol and thymol are not metabolized in anoxic swine waste and they are potentially useful in controlling odor emissions and pathogens in swine waste.

Livestock production and the waste generated can pose a threat to soil, water, and air quality, and to human health. Some of the more serious problems with livestock waste include nutrient enrichment of soil and water, emission of odors and greenhouse gases, as well as presence and transmission of pathogenic microorganisms [16]. Odor and greenhouse gas emissions are a direct result of microbial fermentation of waste [5]. Thus, antimicrobial chemicals may be useful additives to waste to not only control the fermentation, but also destroy the pathogens [15]. Naturally occurring antimicrobial chemicals are desirable [1]. An example of a natural preservative agent is carvacrol, which is present in the essential oil fraction of oregano (60–74%, v/v carvacrol) and thyme (45%, v/v carvacrol) [11]. Thyme also contains a significant amount of thymol (38%) [6], which is an equally effective antimicrobial chemical [2, 15]. These plant-derived oils are generally recognized as safe (GRAS). Carvacrol is routinely used in food production [12], while thymol is used in many different personal care products [6, 10].

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A previous study demonstrated that carvacrol and thymol are effective in controlling odor emissions and pathogens in cattle waste [15]. The objectives in the current study were to determine concentrations of these oils necessary to inhibit the fermentation in swine waste, determine fermentation differences between cattle and swine wastes, and analyze the stability of carvacrol and thymol under anoxic and semioxic conditions.

Materials and Methods

Chemicals. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) with the exception of carvacrol, which was obtained from Aldrich (Milwaukee, WI, USA).

Anoxic and semioxic waste slurries. Swine waste was processed similarly to our previous studies [14, 15]. Fecal waste was randomly collected within 15 min of being excreted from animals fed a finishing diet of 85% corn and 11% soybean meal. Swine urine was collected from catheterized animals. Feces, urine, and distilled water in the ratio 50:35:15 were blended (Waring Inc., New Hartford, CT) for 1 min. Four replicate samples were obtained from this slurry and analyzed for various parameters and were considered as time 0. The waste slurry was divided into 500 ml aliquots and antimicrobial plant oils were added directly at the desired concentration, with one exception, in

which carvacrol was dissolved 1:1 v:v in 95% ethanol, or 2 ml carvacrol/ethanol addition in these 500 ml slurries. The slurry was blended 1 min to provide a homogenous mixing of the antimicrobial oils and poured into 1-liter Erlenmeyer flasks, which were gassed with nitrogen (also after each sampling), sealed with a rubber stopper, and left stationary at ambient temperature (25°C). Treatments were in duplicate, and the contents of the flasks were gently swirled before being sampled at the days indicated in the figures. Gas volume and composition were analyzed in these flasks (anoic slurry). In other experiments, wide-mouth (10 cm) jars (17 cm tall, 13.5 cm in diameter, 1.6-liter volume) as previously described [15] were used to simulate natural lagoon or basin conditions for storage of swine waste (semioxic slurry). Plastic lids covered approximately 90% of the jar opening to prevent moisture loss over the experimental period. The sampling procedure was similar to that described above, except no stirring or mixing occurred before the contents were sampled, and treatments were in triplicate.

Methods of analysis. Head space gas was measured by displacement of a water-lubricated glass piston in a 50 ml syringe [7]. A 20-gauge needle with a Leur-lok was inserted through the stopper, and a three-way valve was attached to the needle to allow gas volume to be periodically determined. Methane and hydrogen were analyzed as previously described [14].

A 15 ml waste sample was collected from each flask or jar. The sample was mixed with 15 ml of 0.5 M H_2SO_4, centrifuged at 2000 × *g* for 20 min at 4°C, and stored at −20°C until analyzed [16]. L-Lactate concentrations were determined with a membrane-immobilized system involving lactate oxidase (Model 27, Yellow Springs Instrument Co., Yellow Springs, OH, USA). Short-chain volatile fatty acids (VFAs; acetate, propionate, butyrate, valerate, isobutyrate, isovalerate) and aromatic compounds (cresol, indole, skatole, 4-ethylphenol, phenol) were determined in an aliquot from the original acidified sample. After thawing, the sample was centrifuged at 5°C, 10,000 × *g* for 5 min. A 0.5 ml aliquot of the supernatant was combined with an internal standard, ethyl butyrate (0.25 mM final concn), sample was acidified with 0.4 ml of 3 M HCl, 0.8 ml ethyl ether was added, sample was shaken vigorously for 1 min, and centrifuged at 5°C, 16,000 × *g* for 1 min, and the ether phase was analyzed. To determine carvacrol and thymol concentrations, an aliquot of the original acidified sample which was not centrifuged, was treated as indicated above and extracted with ether twice. These two chemicals were found to primarily reside in the waste solids. Aromatic, VFAs, thymol, and carvacrol were analyzed with a Hewlett Packard 6890 gas chromatograph equipped with a flame ionization detector and a Hewlett Packard 5973 mass selective detector. Compounds were separated on a 30 m × 0.32 mm diameter (0.5 μm film thickness) Innowax PEG column using the following program parameters:flow rate was 1.9 ml min^{-1}, initial temperature was 140°C, initial time was 3 min with a temperature ramp of 7.5°C min^{-1}, with a final temperature of 230°C for 4 min. Injector and detector temperatures were 250°C.

Total culturable anaerobic bacteria and fecal coliforms were enumerated from a 1-ml sample removed from each jar as previously described [15]. Fecal coliforms were enumerated with 3 M Petrifilm *Escherichia coli* coliform count plates (3 M Microbiology Products, St. Paul, MN).

Statistical analysis. Data were analyzed as a split-plot in time with the GLM procedure of Statistical Analysis System (SAS) [9]. Differences between means were tested with a linear model that included treatment and day as discrete effects. The model was treatment, jar or flask nested within treatment, day, and treatment by day. Treatment means were tested with jar or flask nested within treatment as the source of error. Day and treatment by day means were tested with the residual mean squares as the source of error. Least-square means with standard errors are presented in the figures and tables. Each mean represents triplicate (jars) or duplicate (flasks) samples (n = 3 or 2, respectively).

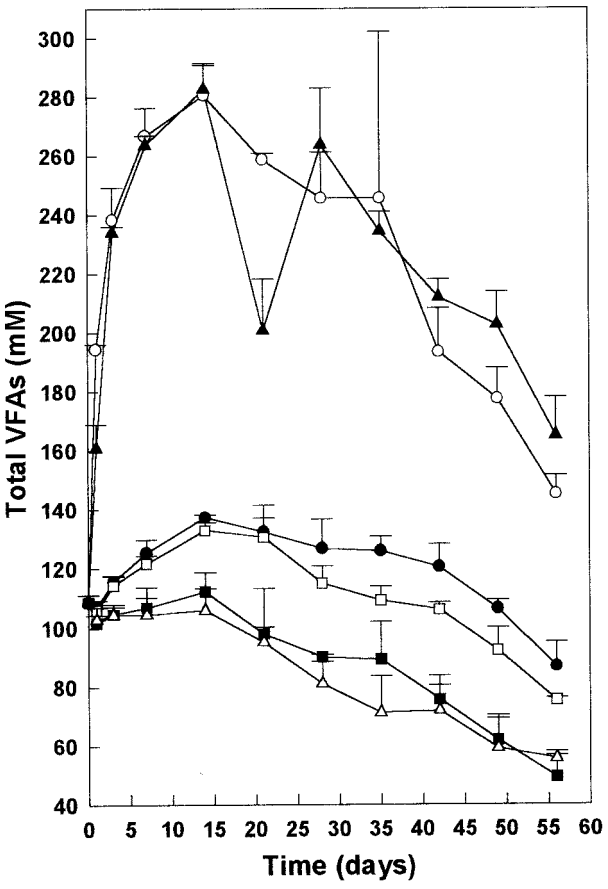

Fig. 1. Effect of various antimicrobial treatments on the production of total short-chain volatile fatty acids (VFAs) from stored swine waste. Treatments included: ○ control (no additions); ● carvacrol 13.3 mM; □ carvacrol 13.3 mM (in 95% ethanol, 1:1); ■ carvacrol 16.75 mM; △ carvacrol 20 mM; ▲ chlorhexidine diacetate 2 mM and iodoacetate 2 mM. Treatment, day, and treatment by day interactions were significant ($p < 0.01$).

Results

An initial experiment was conducted in open jars to determine if the antimicrobial additives, chlorhexidine diacetate and iodoacetate, which were effective with cattle waste [14], and carvacrol, would be effective in controlling odor emissions from stored swine waste. The data in Fig. 1 indicate that a combination of chlorhexidine diacetate (2 mM) and iodoacetate (2 mM) had no inhibitory effect on the production of VFAs from swine waste when compared to control values. However, the three concentrations of carvacrol inhibited the production of VFAs ($p < 0.01$). Dissolving carvacrol in ethanol prior to adding it to the waste had no effect ($p >$

Table 1. Reduction of total anaerobic bacteria in swine waste slurries incubated anaerobically after carvacrol was added at two concentrations

Time (days)	Anaerobic bacteria (10^9 cells per ml)[1]			
	Control	Carvacrol (mM)		
	No additions	13.3	20.0	SE
0	124	124	124	9
2	30.5[a]	0.72[b]	0.003[c]	0.4
7	20.8[a]	0.22[b]	0.001[c]	0.3
14	15.3[a]	0.08[b]	0.001[c]	0.1
28	9.5[a]	0.14[b]	0.001[c]	0.1

[1]Means represent the average from three replicate jars.
[a,b,c]Means in a row with different superscripts differ ($p < 0.05$).

Table 2. Reduction of total fecal coliforms in swine waste slurries incubated anaerobically after carvacrol was added at two concentrations

Time (days)	Coliform bacteria (10^5 cells per ml)[1]			
	Control	Carvacrol (mM)		
	No additions	13.3	20.0	SE
0	63	63	63	4
2	46[a]	0.01[b]	ND[2]	0.01
7	7.4[a]	0.001[b]		0.01
14	2.2	ND[2]		0.5

[1]Means represent the average from three replicate jars.
[2]Not detectable; detection limit is $\geq 1.0 \times 10^2$.
[a,b]Means in a row with different superscripts differ ($p < 0.01$).

0.05) on its ability to inhibit VFA production; thus, it was not dissolved in subsequent experiments.

Total culturable anaerobic bacteria and fecal coliforms were enumerated from the control, 13.3 mM, and 20 mM carvacrol treatments. Both of the carvacrol treatments reduced ($p < 0.05$) the number of viable anaerobic bacteria in the waste within 2 days when compared to the controls (Table 1). This effect was sustained for 28 days. The initial decrease in the concentrations of anaerobic bacteria from day 0 to 2 in the control is presumably because VFAs increase in this batch system and become lethal to a select population of organisms. No fecal coliforms were detected after 2 days when 20 mM carvacrol was added to the waste (Table 2). The 13.3 mM carvacrol treatment reduced ($p < 0.01$) the fecal coliforms within 2 days, and none were detected at 14 days.

Sealed flasks were used to validate whether carvacrol and thymol were equally effective in controlling fermentation activity in swine waste, to further explore the inhibited products, and determine the stability of the additives (Fig. 2A–D; Fig. 3A–B). Carvacrol and thymol at 13.3 or 16.75 mM, or a combination of each to equal 16.75 mM, essentially stopped most gas production (Fig. 2A) and prevented any production of the offensive odor compounds valerate, isovalerate, isobutyrate, and cresol (data not shown). Also, production of propionate was inhibited and only a minimum of butyrate (< 8 mM) was produced in these treatments (data not shown). Acetate was the predominant acid that increased and was responsible for the increase in total VFA production (Fig. 2B). The gas composition (data not shown) from the control and combination thymol/carvacrol treatments (13.3 mM) was primarily methane, with traces of hydrogen (carbon dioxide was not measured). Trace amounts of methane were also detected after 20 days in the carvacrol and thymol treatments (13.3 mM). Lactate accumulated only when carvacrol or thymol were added to the waste; however, lactate decreased between day 10 and 20 in the 13.3 mM treatments (Fig. 2C). The pH decreased in the control and all treatments during the first 2 days (Fig. 2D); however, with the exception of the 16.75 mM carvacrol, thymol, and combination thymol/carvacrol treatments, pH rose with time which corresponded to the initiation of methane production and the disappearance of lactate (Fig. 2C).

In general, less than 25% of the added thymol was recovered from the liquid or supernatant fraction of swine waste (Fig. 3A). However, from the solid fraction of the waste, 90 to 95% of the added thymol was recovered (Fig. 3B). The recoveries were similar for carvacrol from sealed flasks. Also, both oils were recovered from open jars after 56 days at 90 to 95% of added concentrations, indicating that these aromatic chemicals did not volatilize from open vessels (data not shown).

Discussion

It is our hypothesis that if we inhibit the production of fermentation gas and short-chain VFAs in stored livestock waste, less odor will be emitted from these wastes [15]. This is supported by the studies of Zahn et al. [18] in which they conclude that C2 through C9 organic acids from swine waste demonstrate the greatest potential for decreased air quality, since these compounds exhibit the highest transport coefficients and highest airborne concentrations.

Our earlier study indicated that a combination of chlorhexidine diacetate and iodoacetate added to stored cattle waste reduced the production of VFAs by 50% [14]. However, when these chemicals were added to swine waste, no inhibitory effect was observed (Fig. 1). This suggests that the microbial populations in the two wastes are different. This is also supported by the data in Fig. 2C which indicates only small concentrations (< 5

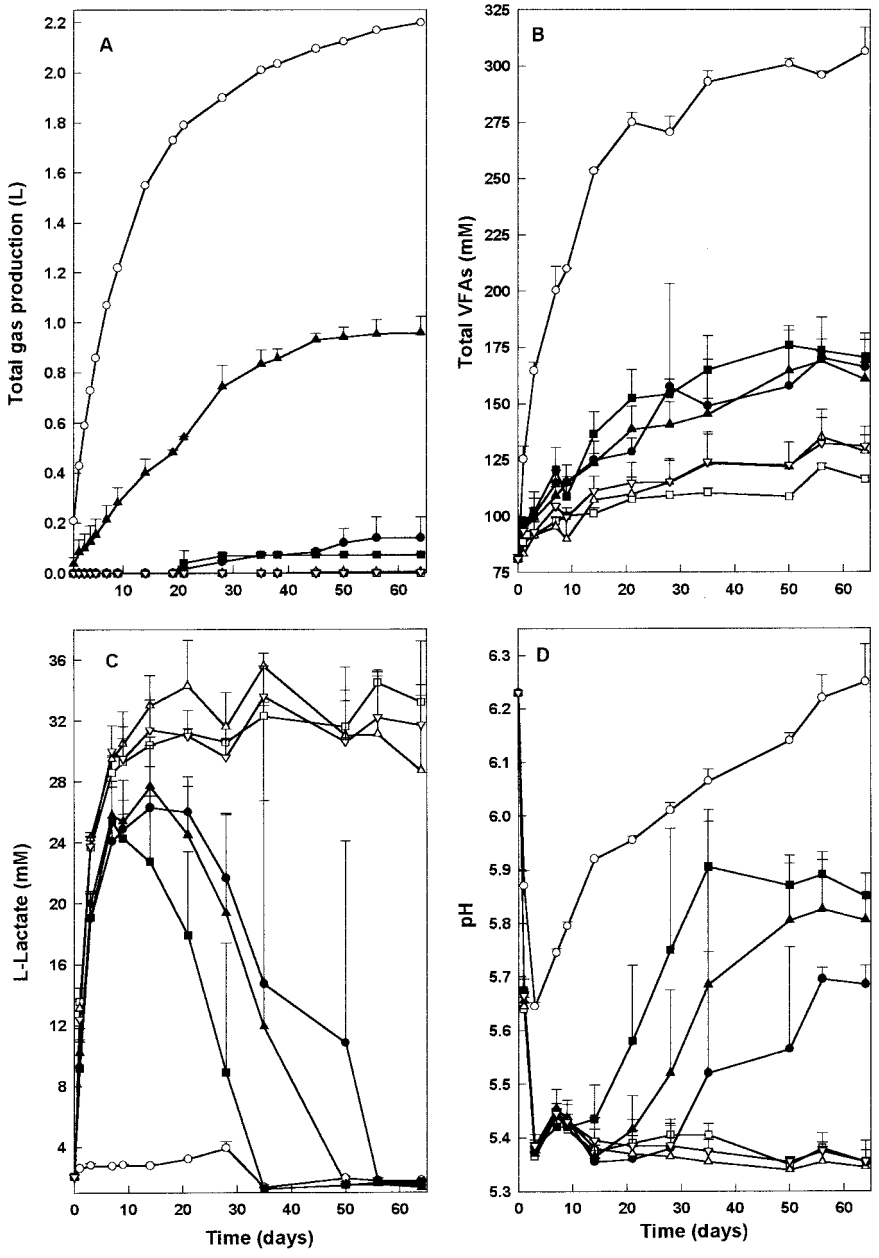

Fig. 2. Effect of thymol and carvacrol treatments on gas production. VFAs, lactate, and pH in stored swine waste. Treatments included: ○ control (no additions); ● thymol 13.3 mM; □ thymol 16.75 mM; ■ carvacrol 13.3 mM; △ carvacrol 16.75 mM; ▲ thymol 6.7 mM and carvacrol 6.7 mM; ▽ thymol 8.35 mM and carvacrol 8.35 mM. Treatment, day, and treatment by day interactions were significant ($p < 0.01$).

mM) of lactate accumulate in untreated swine waste, whereas high concentrations (200 mM) will accumulate in untreated cattle waste [14]. In the current study, lactate accumulated in swine waste only when carvacrol or thymol were added. This indicates that these antimicrobial agents either inhibit a group of microorganisms whereby the lactate producing microorganisms are able to compete, or these agents suppress microorganisms that utilize lactate.

In agreement with our cattle waste study [15] carvacrol and thymol inhibited most microbial fermentation activity in swine waste, significantly reduced the anaerobic microorganisms, and eliminated the fecal coliforms; although a slightly higher concentration (3.5 mM) of these antimicrobial agents may be required in swine waste. In cattle waste, 13.3 mM carvacrol or thymol suppressed essentially all fermentation activity in a waste slurry of feces, urine, and water (50:35:15). However, with swine waste at this same ratio, 16.75 mM was required to suppress most fermentation activity.

The data in Fig. 3A and B indicate that 90 to 95% of the thymol is extracted from the waste solids, which

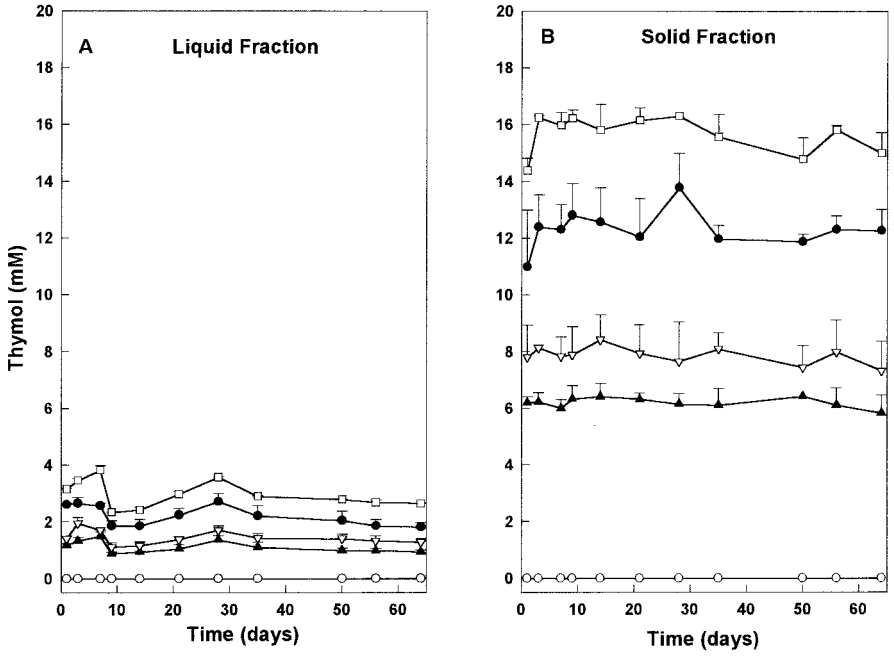

Fig. 3. Recovery of thymol from the liquid and solid fractions of stored swine waste treated with thymol and carvacrol. Treatments were: ○ control (no additions); □ thymol 16.75 mM; thymol 13.3 mM; ▽ thymol 8.35 mM; ▲ thymol 6.7 mM. Carvacrol concentrations are not shown but they were similar to thymol.

supports the idea that these agents bind to organic matter. Kim et al. [4] found that a 1.5% solution of carvacrol was necessary to kill *Streptococcus enterica* serovar *typhimurium* on fish cubes, a level which is considerably higher than the concentration (0.1%) needed to kill the organism in a low organic matter broth medium. The organic matter in the cattle and swine wastes used in this study was similar (96.8 and 96.9%, respectively). This suggests that total organic matter is not primarily responsible for the higher concentration of carvacrol or thymol needed to suppress microbial activity in swine waste, but the differing microbial populations are responsible.

Gas was produced from waste treated with 13.3 mM carvacrol, or thymol, or a combination of the two (Fig. 2A). Methane was the predominant gas, indicating that these chemicals are not bactericidal to the methanogenic population. Previous studies have suggested that a combination of both oils would provide better antimicrobial action rather than a higher concentration of carvacrol or thymol alone [6, 8]. Other parameters (Fig. 2 B,C) indicated essentially no difference whether the individual chemical was added or a combination of the two was added to equal 13.3 mM.

Ultee et al. [11] have recently found that the foodborne pathogen, *Bacillus cereus*, adapts to carvacrol when present at non-lethal concentrations by lowering its membrane fluidity. However, carvacrol was not metabolized by the organism. The results here (Fig. 3A,B) suggest that mixed microbial populations in swine waste do not metabolize thymol (or carvacrol) for at least 62 days under anoxic conditions. Soil microorganisms are known to degrade some of the monoterpenoid plant essential oils [13] and some are also degraded under anaerobic conditions [3]. Vokou and Liotiri [17] have concluded that essential oils are used as a carbon and energy source by ubiquitously occurring soil microorganisms and they would not accumulate in soil if environmental conditions favor growth of these microorganisms. This suggests that carvacrol and thymol used in our treatments will be degraded once the swine waste is applied to soil as a fertilizer.

Conclusions

Laboratory results show that the plant-derived oils, carvacrol and thymol (16.75 mM or 2.5 g/l) can be used to inhibit the microbial fermentation of stored swine waste. This reduces gas and odor emissions from the waste and eliminates the pathogenic fecal coliforms. Further studies are needed to investigate the effectiveness of these additives in livestock production facilities, the economics, and the potential of any adverse environmental effects of these additives when the waste is used as fertilizer.

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