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Plant-Derived Oils Reduce Pathogens and Gaseous Emissions from Stored Cattle Waste

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Carvacrol and thymol in combination at 6.7 mM each completely inhibited the production of short-chain volatile fatty acids and lactate from cattle waste in anoxic flasks over 23 days. Fecal coliforms were reduced from 4.6×10^6 to 2.0×10^3 cells per ml 2 days after treatment and were nondetectable within 4 days. Total anaerobic bacteria were reduced from 8.4×10^{10} to 1.5×10^7 cells per ml after 2 days and continued to be suppressed to that level after 14 days. If the concentration of carvacrol or thymol were doubled (13.3 mM), either could be used to obtain the same inhibitory fermentation effect. We conclude that carvacrol or thymol may be useful as an antimicrobial chemical to control pathogens and odor in stored livestock waste.

Odor emitted from wastes results from incomplete anoxic degradation of the carbohydrate, protein, and lipid components (11, 27). This incomplete degradation results in the formation of offensive short-chain volatile fatty acids (VFAs), aromatic chemicals, amines and other nitrogenous compounds, and sulfur-containing compounds. In most livestock production facilities it is not possible to control the environment in which complete degradation of the waste to methane and carbon dioxide occurs. Conventional anoxic digesters for production of methane were popular during the 1970s and 1980s; however, economics and the technical expertise required to operate these digestors have diminished their popularity (14). Similarly, oxic treatment is not economically feasible, and it does little to conserve nutrients.

We have previously reported that chlorhexidine diacetate, in combination with iodoacetate or diphenyliodonium chloride, can be used to inhibit key anoxic-degradation pathways (26). Another approach to reduce emissions might be the use of naturally occurring antimicrobial chemicals such as plant-derived oils (1–3, 7, 23). Numerous reports indicate that plant oils have antimicrobial activity; however, most of these studies evaluate one oil against one microorganism in an artificial medium (3). Few studies are available that determine the effect of the oils in natural highly diverse microbial ecosystems such as those found in the gastrointestinal tract (15), food products (7, 9, 21), or waste management systems. In this study we evaluated carvacrol (5-isopropyl-2-methylphenol) and thymol (5-methyl-2-isopropylphenol) for their ability to reduce the production of gas and short-chain VFAs and the viability of total anaerobic bacteria and fecal coliforms in stored cattle waste.

Waste was collected and processed similarly to our previous study (26). The waste slurry was divided into 500-ml aliquots, and antimicrobial plant oils were added directly to the slurry at the desired concentration. The slurry was blended 1 min to provide a homogenous mixing of the antimicrobial oils and poured into a 1-liter Erlenmeyer flask, which was sealed with a rubber stopper and left stationary at ambient temperature (25°C). Treatments were in triplicate, and the contents in the flasks were gently swirled before being sampled at the times indicated (Fig. 1 and Table 1). Another study evaluated the effect of the antimicrobial plant oils on cattle waste under natural semioxic conditions (lagoon or basin simulation). In this study wide-mouth (10-cm) jars (17 cm tall, 13.5 cm in diameter, 1.6-liter volume) with a working volume of 1 liter each were used (Fig. 2). Plastic lids provided with the jars were used to cover approximately 90% of the jar opening to prevent moisture loss over the 56-day experimental period. The sampling procedure was similar to that described above, except that no stirring or mixing occurred before the contents were sampled. A wide-mouth pipette was inserted into the slurry from top to bottom at which time slurry contents were simultaneously withdrawn. Gas volume and composition, short-chain VFAs, and L-lactate in the flasks were measured as previously described (13, 18, 26).

Total culturable anaerobic bacteria and fecal coliforms were enumerated from a 1-ml sample removed from each flask. Each sample was serially diluted in anaerobic buffer, and triplicate roll tubes from each of three dilutions containing 30% ruminal fluid-based medium were inoculated as previously described (25). Roll tubes were incubated at 35°C, and colonies were counted after 7 days. Fecal coliforms were enumerated with 3M Petrifilm *Escherichia coli* coliform count plates (3M Microbiology Products, St. Paul, Minn.). Triplicate plates for each of three dilutions were inoculated and incubated at 35°C, and colonies were counted using AOAC official methods as described in the literature provided with the plates. Briefly, total coliform numbers consisted of both red and blue colonies associated with gas at 24 h after inoculation. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) with the exception of carvacrol, which was obtained from Aldrich (Milwaukee, Wisc.). Data were analyzed as a split-plot in time with the general linear models (GLM) procedure of SAS Inst., Inc. (Gary, N.C.) (19).

A series of experiments using different batches of waste and

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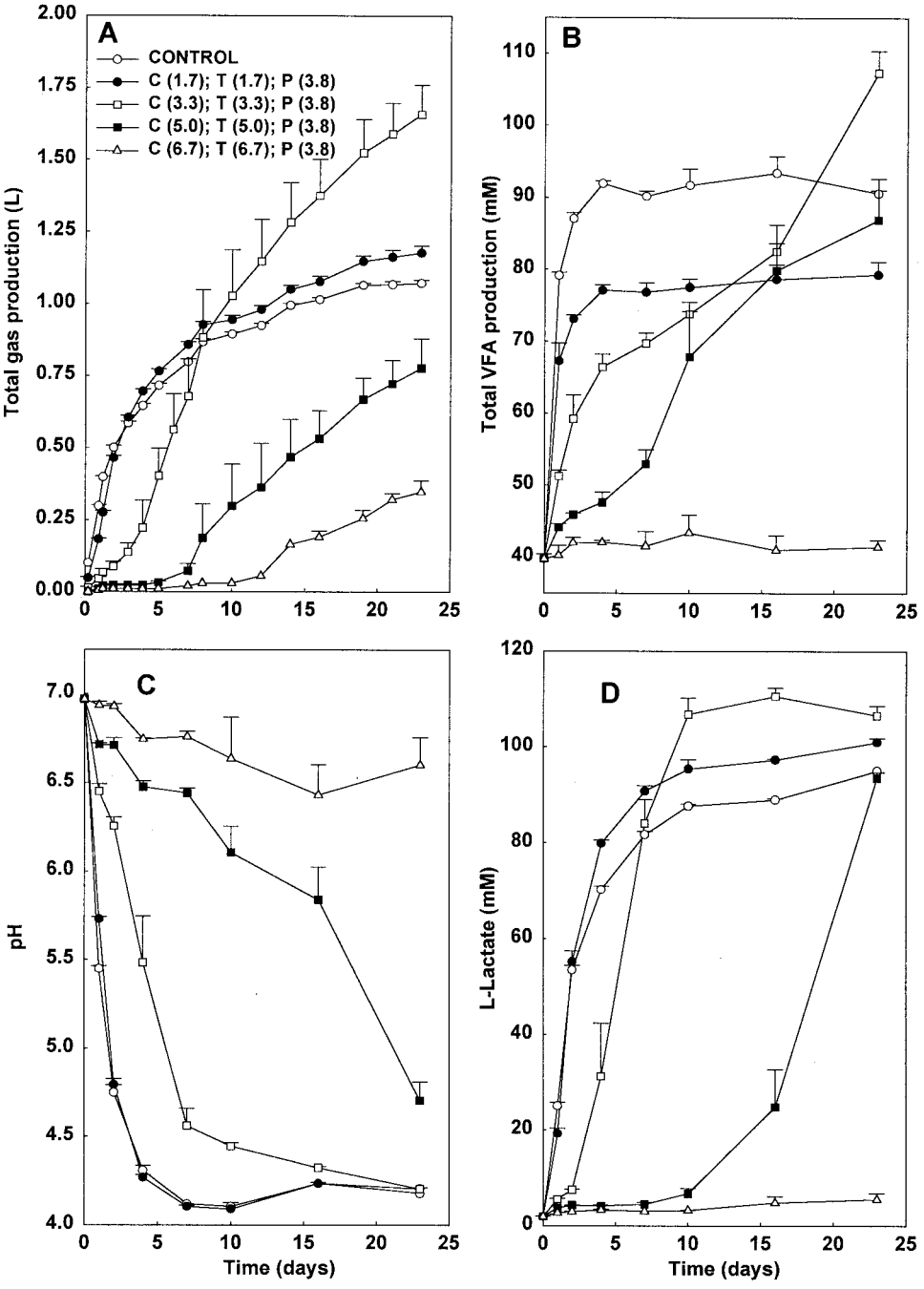

FIG. 1. Effect of various concentrations of carvacrol, thymol, and pinene on the production of gas, total short-chain VFAs, pH, and L-lactate in stored cattle waste. Control, no additions; C, carvacrol; T, thymol; P, α-pinene. Numbers in parentheses in legend are millimolar concentrations. Treatment, day, and treatment by day interactions were significant ($P < 0.01$).

different time periods with plant-derived oils was conducted to evaluate the oils individually or in combination, for their ability to control the production of short-chain VFA, L-lactate, and gas in stored cattle waste. Oils that were not effective in controlling acid production included the following, with the millimolar concentration of the treatment and total VFAs given first, followed by total VFAs in the respective controls after the slash: 7.5 mM α-pinene, 105/110; 3.8 mM each α-pinene and limonene 166/195; 3.3 mM camphor, 175/195, 6.7 mM geraniol, 75/85; and a combination of 3.8 mM each α-pinene and limonene, 3.3 mM camphor, and 2.0 mM each borneol and fenchol, 86/92. Thymol and carvacrol were most effective in the initial screenings, and only results from these two oils are presented. Pinene was added as an odor-masking agent with the thymol and carvacrol treatments but was not added to the control flasks or jars. Pinene had little antimicrobial activity, as indicated above and in a previous report (26).

Data in Fig. 1(A to D) indicated that a combination of

TABLE 1. Reduction of total anaerobic bacteria and fecal coliforms in cattle waste slurries incubated anaerobically after addition of carvacrol, thymol, and pinene at two concentrations

Time, days	Anaerobes (10^8 cells/ml)[a]/coliforms (10^5 cells per ml)[a]			
	Control	C, T (5.0 mM each); P (3.8 mM)	C, T (6.7 mM each); P (3.8 mM)	SE
0	844/46	844/46	844/46	56/5.1
2	79.4*/81*	4.0†/1.5†	0.15‡/0.02‡	0.8/0.4
4	NE/2.9*	NE/0.44†	NE/ND	NE/0.3
7	78.3*/1.0	1.4†/0.57	0.06‡/NE	0.5/0.4
14	33.6*/ND	7.6†/0.03	0.16‡/NE	0.9/0.005

[a] Means represent the average from three replicate flasks. Means in a row with different superscripts (*, †, ‡) differ ($P < 0.05$). NE, not enumerated. ND, none detected (detection limit, $\geq 10^2$).

6.7 mM carvacrol, 6.7 mM thymol and 3.8 mM pinene inhibited most microbial activity for 23 days in anoxic flasks containing cattle waste. After approximately 7 days, some gas production was observed at this treatment concentration (Fig. 1A); however, no increases in total volatile fatty acids (Fig. 1B) or lactate (Fig. 1D) were observed. The pH data also supported an inhibition of acid production or fermentation activity because it remained between 6.5 and 7 (Fig. 1C). The gas that was produced was most likely CO_2 (not analyzed), because no CH_4 and only traces of H_2 were detected. The treatment with 3.3 mM carvacrol, 3.3 mM thymol, and 3.8 mM pinene suggests that several metabolic groups of microorganisms may be inhibited, which in turn allows others to flourish and produce more gas, lactate, and total VFAs. Acetate was responsible for the increase in total VFAs, since no additional propionate or butyrate was observed.

Data in Table 1 indicate that a combination of carvacrol, thymol, and pinene at both concentrations evaluated, 5 mM or 6.7 mM each for carvacrol and thymol, significantly reduced the number of viable anaerobic bacteria in the waste within 2 days when compared with that in the control flasks. We did not see a complete bactericidal effect after 14 days, even at the 6.7 mM concentrations of carvacrol and thymol; however, the number of organisms remained low, similar to that in the 2-day population. The population of fecal coliforms was reduced to nondetectable levels after 4 days when carvacrol and thymol were combined at 6.7 mM each (Table 1). The absence of fecal coliforms in the control flasks after 14 days was likely due to acidification, since pH dropped to 4.2 after 14 days (Fig. 1C).

In a second group of incubations, open wide-mouthed jars were used to determine whether carvacrol or thymol could be used in combination or individually to inhibit microbial activity in semioxic waste. The open containers were used to more closely mimic the conditions in a lagoon where cattle feedlot waste would be stored. The data in Fig. 2A indicate that carvacrol or thymol individually at 13.3 mM inhibited production of short-chain VFAs equally or better than the combination of the two oils (6.7 mM each) for 56 days. The control treatment exhibited a biphasic production of VFAs (Fig. 2A). There was an initial rapid production of VFAs up to day 4 and then another increase in VFAs beginning at day 14. In the control jars a crust began to form on top of the waste, which may have contributed to the secondary fermentation. Although acetate (40 mM) and propionate (25 mM) were produced, the predominant acid produced during this secondary fermentation (days 14 to 42) was butyrate (100 mM [Fig. 2B]). Significant gas bubbles were produced beginning at days 12 to 14, which raised

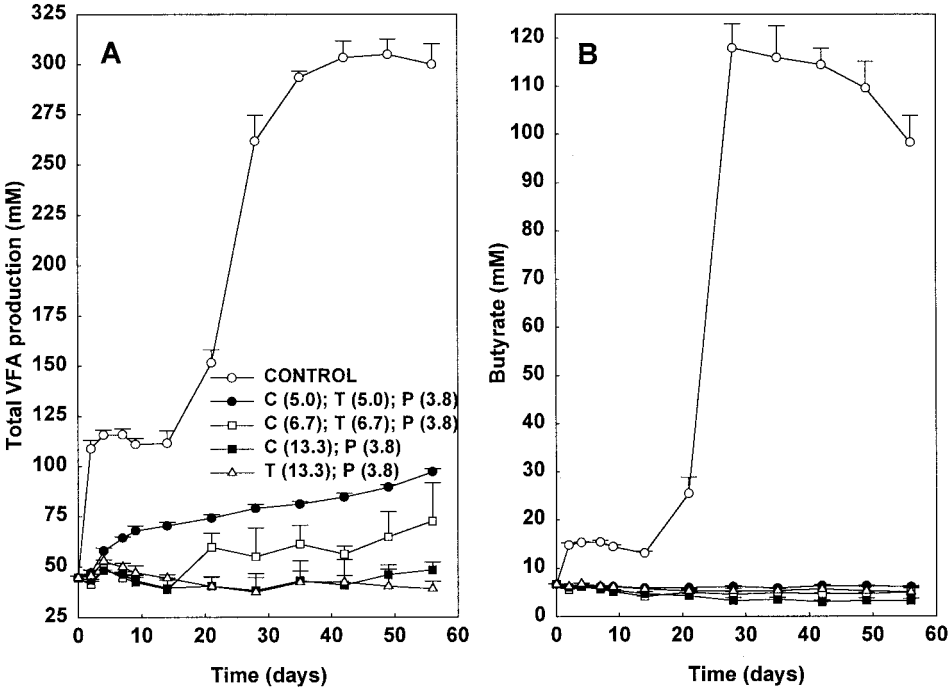

FIG. 2. Effect of various concentrations of carvacrol, thymol, and pinene on the production of total short-chain VFAs and butyrate in stored cattle waste. Control, no additions; C, carvacrol; T, thymol; P, α-pinene. Numbers in parentheses in legend are millimolar concentrations. Treatment, day, and treatment by day interactions were significant ($P < 0.01$).

the crust to the top of the jar. Because of the high concentration of butyrate, we believe that a significant amount of the gas that was produced was hydrogen, resulting from a classic *Clostridium butyricum* fermentation which we have previously observed (26). The crust did not form on any of the treated wastes.

The results from this study suggest that a combination of carvacrol (6.7 mM) and thymol (6.7 mM) or each individually at 13.3 mM will stop most fermentation activity in stored cattle waste. Previous studies have suggested that a combination of both oils would provide better antimicrobial action, rather than a higher content of carvacrol or thymol alone (12, 16). Although pinene was added to the treatments (not the controls), it has little inhibitory activity (26). We observed that it serves as an effective odor-masking agent in anoxic flask fermentations but not in the semioxic jar, where it must rapidly volatilize.

Numerous studies show that carvacrol and thymol are bactericidal to pathogens (5, 9, 17, 22, 23) and in particular to *E. coli* O157:H7 (7, 10, 21) in pure cultures. Helander et al. (7) have shown that the MIC with carvacrol or thymol in a pure culture system is 3 mM and 1 mM for *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium, respectively. Kim et al. (10) also have found that 500 μg of carvacrol/ml (3.3 mM) will kill *E. coli* O157:H7. We found that a combination of carvacrol and thymol appears to be bactericidal to fecal coliforms (Table 1). However, our findings illustrated that concentrations must be doubled for bactericidal activity in the highly organic, mixed microbial community found in animal waste compared to the concentrations needed to kill pathogens in pure culture.

Because animal wastes normally have a high concentration of organic matter some of the hydrophobic carvacrol or thymol may simply bind to the organic matter; thus, a higher concentration of these oils may be needed to supply a surplus needed to enter into microbial cell membranes. This observation also challenges the validity of results from antimicrobial studies which evaluate one organism in minimal medium compared to organisms in the natural ecosystem. In vivo studies are needed to confirm the validity of the results from various in vitro studies (5, 8). Kim et al. (9) found that a 1.5% solution of carvacrol was necessary to kill *S. enterica* serovar Typhimurium on fish cubes, a level which is significantly higher than the concentration (0.1%) needed to kill the organism in liquid medium.

Control of pathogenic *E. coli* at animal production facilities has become a research priority. Elder et al. (4) have found that 28% of cattle may be carriers of *E. coli* O157 in their feces, and 11% of cattle presented for slaughter carry *E. coli* O157 on their hides. They conclude that there is a correlation between fecal prevalence and carcass contamination. One plausible way to limit the prevalence of *E. coli* O157:H7 in feedlot cattle is to limit the exposure of uninfected cattle in the feedlot to this organism. Waste treated with carvacrol or thymol may reduce the spread of *E. coli* O157:H7 among cattle in the same or adjacent pens and could potentially reduce the percentage of infected cattle and hide contamination at slaughter. More sensitive and specific evaluations are currently being conducted to determine whether *E. coli* O157:H7 is specifically eliminated from feces and soil in cattle feedlot pens.

We hypothesize that if the production of fermentation gas and short-chain VFAs is inhibited in stored livestock waste, less odor will be emitted from these wastes. This is supported by the studies of Zahn et al. (28) 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. In our study, carvacrol and thymol inhibited most fermentation activity in the stored waste, and the pH remained neutral (6.5 to 7.0), which kept the short-chain VFAs in the ionized, less volatile state.

Whether concentrations of the oils used in our study to kill fecal pathogens in their natural habitat or to control odor emissions are safe in a livestock production system remains to be determined. In practice, carvacrol is added to different products, such as baked goods (16 ppm), nonalcoholic beverages (28 ppm/0.18 mM), and chewing gum (8 ppm) (23). Thymol is a component in many different products including soaps, toothpastes, shampoos, deodorants, and mouthwashes (12, 20). These chemicals, like most plant-derived oils, are generally recognized as safe; however, any chemical can be environmentally unsafe when used in high concentrations. It is feasible that some carvacrol or thymol would volatilize from these wastes or be metabolized by soil microorganisms (6, 24).

In conclusion, we feel that carvacrol or thymol could be used as an additive to stored livestock waste to reduce odor emissions, global-warming gases, and pathogens, which should in turn retain nutrients or organic matter in the waste and enhance the fertilizer value. Crops or produce obtained from land which has been fertilized with treated waste is likely to carry fewer food-borne pathogens than crops or produce obtained from land fertilized with untreated waste. The economics and environmental effects of using carvacrol or thymol at the concentrations used in this study need to be determined.

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