Journal of Food Protection, Vol. 62, No. 9, 1999, Pages 975–979

Effect of Diacetyl on Controlling *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in the Presence of Starter Culture in a Laboratory Medium and during Meat Fermentation

DONG-HYUN KANG* AND DANIEL Y. C. FUNG

Food Microbiology Laboratory, Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas 66506-1600, USA

MS 98-240: Received 4 September 1998/Accepted 8 March 1999

ABSTRACT

Diacetyl is a flavor compound that possesses antimicrobial activity and is found in several dairy products. The effect of diacetyl on controlling the growth of two foodborne pathogens, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium, when grown with *Pediococcus acidilactici* as a meat starter culture was evaluated in a laboratory medium and during salami fermentation. Diacetyl (50 ppm) added to each mixed culture system strongly inhibited the growth of *E. coli* O157:H7 and *Salmonella* Typhimurium in the laboratory medium (brain heart infusion, 2.3% of NaCl, 0.75% of dextrose) ($P < 0.05$). During meat fermentation, the growth of *E. coli* O157:H7 and *Salmonella* Typhimurium was inhibited significantly by addition of diacetyl (300 ppm) ($P < 0.05$) after 24 h fermentation. However, the acid production and growth of *P. acidilactici* were not affected by the addition of diacetyl ($P > 0.05$). After 24 h meat fermentation, about a 1.0-log CFU/g difference occurred in numbers of each foodborne pathogen mixed with *P. acidilactici* ($P < 0.05$) with and without 300 ppm diacetyl. Diacetyl and the acid produced by the meat starter culture reduced the growth of the two foodborne pathogens during salami fermentation. These results suggest that diacetyl can be used as a food ingredient during meat fermentation to control *E. coli* O157:H7 and *Salmonella* Typhimurium without harmful effects on the growth and acid production of *P. acidilactici.*

Escherichia coli O157:H7 and *Salmonella* Typhimurium are important foodborne pathogens in meat systems *(4, 12, 17, 19, 20, 22)*; several outbreaks have been associated with fermented meat products *(2, 3, 5, 6, 8).* Many food-grade ingredients have been reported to stimulate starter cultures or inhibit those foodborne pathogens during meat fermentation *(4, 8, 14).* Among those ingredients, diacetyl was identified as a flavor compound in butter in 1929 *(21).* It also has antimicrobial activity against several gram-negative microorganisms such as *Escherichia coli (10, 11)* and *Salmonella (1). Streptococcus, Leuconostoc, Lactobacillus,* and *Pediococcus,* as well as other microorganisms, produce diacetyl *(13).* Most studies have evaluated the antimicrobial activities of diacetyl in pure culture systems or pathogen-inoculated food systems *(1, 10, 11, 13).* Furthermore, most research has focused on dairy products such as butter, cheese, or skim milk *(1, 15, 16).* In fermented meat products, such as salami, starter cultures produce acid. To date, the application of diacetyl to meat systems has not been reported and evaluated. Jay *(13)* reported that the combination of acidic conditions and diacetyl decreased the numbers of foodborne pathogens. The effect of diacetyl on acid production and growth of starter cultures should also be considered. Several papers have reported that diacetyl has little antimicrobial activity against lactic acid bacteria compared to gram-negative bacteria *(1, 10, 15, 16).* The relationship between meat components such as fat and the activity of diacetyl is also important. The objective of this experiment was to determine the effect of diacetyl on growth and destruction of *E. coli* O157:H7 and *Salmonella* Typhimurium mixed with starter culture (*P. acidilactici*) in a laboratory medium and during meat fermentation.

* Author for correspondence. Present address: U.S. Department of Agriculture, Agricultural Research Service, Roman L. Hruska U.S. Meat Animal Research Center, P.O. Box 166, Clay Center, NE 68933, USA. Tel: 402-762-4381; Fax: 402-762-4149; E-mail: kang@email.marc.usda.gov.

MATERIALS AND METHODS

Diacetyl. One milliliter of diacetyl (Sigma Chemical Co., St. Louis, Mo.) was mixed completely in 100 ml of sterilized water. The mixture was passed through a 0.2-μm membrane filter to obtain sterilized filtrates. The solution was stored in the refrigerator and used as a stock solution for further research.

Cultures. *P. acidilactici* isolated from HP starter culture (Diversitech Inc., Duncanville, Tex.) was transferred into brain heart infusion broth (BHI; Difco, Detroit, Mich.) and incubated at 37°C for 48 h. *E. coli* O157:H7 (Eh7–7) and *Salmonella* Typhimurium (ATCC 6994) were obtained from the Food Microbiology Culture Collection at Kansas State University and transferred into BHI broth. The cultures then were incubated at 37°C for 24 h. Lactobacilli MRS (Difco) medium for *P. acidilactici,* sorbitol MacConkey agar (Difco) for *E. coli* O157:H7, and brilliant green agar (Difco) for *Salmonella* Typhimurium were used to enumerate each microorganism. Inoculated MRS medium was incubated at 37°C for 48 h in a Brewer anaerobic jar (BBL Gas Pak System, Becton Dickinson and Co., Sparks, Md.) with a CO_2 catalyst (BBL). Sorbitol MacConkey agar and brilliant green agar were incubated aerobically at 37°C for 24 h.

Effect of diacetyl on control of *E. coli* O157:H7 and *Salmonella* Typhimurium mixed with *P. acidilactici* in a labora-

tory medium. *E. coli* O157:H7 or *Salmonella* Typhimurium (~4.00–5.00 log CFU/ml) mixed with *P. acidilactici* (~7.00 log CFU/ml) was inoculated into laboratory medium (pH 6.0, LBHI; BHI + 2.3% NaCl + 0.75% dextrose) with 50 ppm diacetyl (LBHID; LBHI + diacetyl) and without 50 ppm diacetyl (LBHI). Flasks containing inoculated media were incubated without agitation at 40°C for 16 h. Viable cell counts and pH of the broth were measured just before and after 4, 8, 12, and 16 h of incubation using a model D spiral plater (Spiral Systems Instruments, Bethesda, Md.) and a pH meter (Accumet 620, Fisher Scientific, Pittsburgh, Pa.), respectively. Injured microorganisms were not evaluated in this experiment. The experiments were performed in triplicate.

Effect of diacetyl on control of *E. coli* O157:H7 and *Salmonella* Typhimurium by *P. acidilactici* during meat fermentation. An HP starter culture (*P. acidilactici*) obtained from Diversitech Inc. was used for fermentation. *E. coli* O157:H7 (Eh7-7) and *Salmonella* Typhimurium (ATCC 6994) were used independently as the foodborne pathogens. Cultures were incubated on BHI agar slants at 37°C for 24 h. Each pathogen was transferred to 9.0 ml of BHI broth and incubated at 37°C for 24 h. After incubation, 1 ml of each culture broth was transferred into a sterilized 500-ml polycarbonate centrifuge bottle (Nalgene, Rochester, N.Y.) containing 300 ml of BHI broth, and the bottle was incubated at 37°C for 24 h. Cells were harvested by centrifugation (model JA-22A, International Equipment Co., Needhan Heights, Mass.) at 12,400 × *g* for 25 min at 4°C. After initial centrifugation, the pelletized cells were resuspended in 0.1% sterile peptone water, centrifuged, and resuspended in 0.1% sterile peptone water.

Ground beef (90% lean, 10% fat) was obtained from the Meat Laboratory at Kansas State University (Manhattan, Kans.) and used to prepare salami samples. Ground meat was transferred to a ribbon mixer (model I200DA70, Leland South West, Fort Worth, Tex.), to which salt (2.3%) and dextrose (0.75%) were added. To evaluate the effect of diacetyl on the growth of pathogens, only two ingredients (salt and sugar) were used. Dextrose is an important ingredient to reduce pH by starter culture *(14).* Salt is an important ingredient for extraction of myosin from meat. Other spices and nitrate were not added, because they might have affected the growth of foodborne pathogens.

The culture suspensions of *E. coli* O157:H7 and *Salmonella* Typhimurium grown for 24 h at 37°C were diluted serially in 0.1% sterile peptone water. A preliminary study was performed with meat batter to establish dilution levels required to achieve about 6.00- to 7.00-log CFU/g inoculum levels of each pathogen in the meat. The inoculum was pipetted dropwise over the entire surface area of the meat. The meat (3 lb) was mixed by gloved hand massage for 5 min to distribute each pathogen uniformly in the product and then separated into five parts and treated as follows: (i) starter culture (S) + *E. coli* O157:H7 (E) or *Salmonella* Typhimurium (T), (ii) S + E or T + 50 ppm of diacetyl, (iii) S + E or T + 100 ppm of diacetyl, (iv) S + E or T + 200 ppm of diacetyl, and (v) S + E or T + 300 ppm of diacetyl. The 300 ppm was used as the maximum amount of diacetyl that could be added without producing odor in the meat system. The five treated meat batters were stuffed separately into casings. The weight of each salami was about 100 g/casing (2.5 cm diameter and 7.5 cm length).

Salamis were placed in an incubator and fermented for 24 h at 40°C. Samples were taken after 24 h fermentation and were cut using a knife dipped in 70% ethanol and flamed for 2 s *(9).* After meat was diluted 10^{-1} with sterilized water, the pH of five samples for each pathogen-inoculated product was measured with a pH meter *(14).* For viable cell counts, 25 g of each sample were added to 225 ml of 0.1% peptone diluent in a stomacher bag (model SFB 0410, Spiral Biotech Inc., Bethesda, Md.) and homogenized with a stomacher (model 400, Seward Medical, London, UK) for 2 min. The samples were serially diluted 10-fold and plated onto MRS (*P. acidilactici*), sorbitol MacConkey agar (*E. coli* O157:H7), and brilliant green agar (*Salmonella* Typhimurium). These experiments were performed three times.

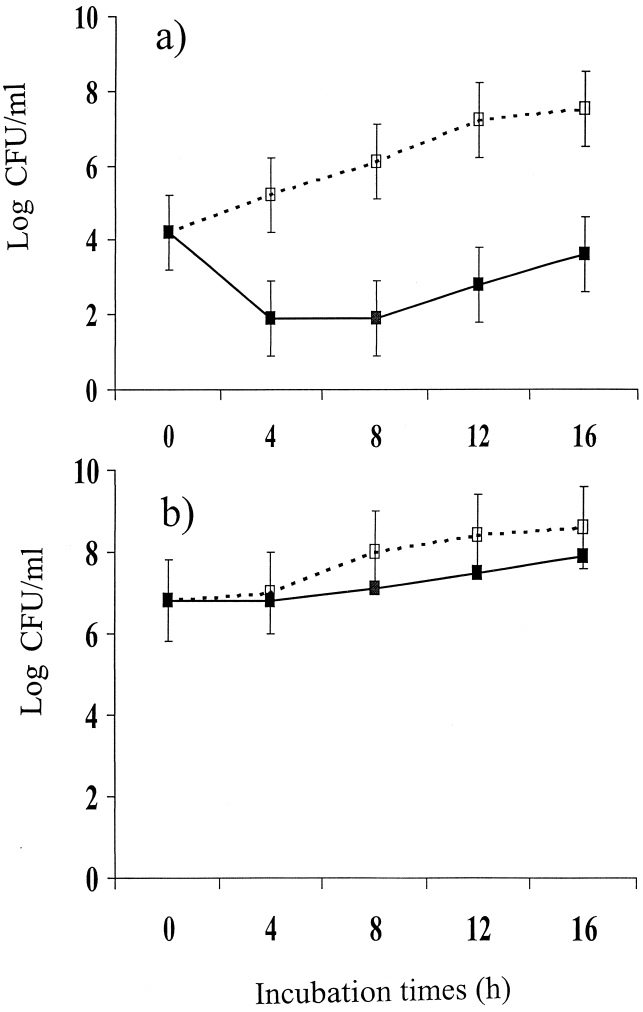

FIGURE 1. *Effect of diacetyl on the growth of mixed cultures of* E. coli *O157:H7 (a) and* P. acidilactici *(b) in a laboratory medium with (■) or without 50 ppm diacetyl (□).*

Statistical analysis. Analysis of variance was performed on cell numbers and pH values using the general linear model procedure of SAS *(18).* The means of three replicates were plotted in graphs and table. Cell counts were converted into logarithm values to determine the significance of differences at the 95% confidence limit ($P = 0.05$). Differences among treatments were examined for levels of significance by Duncan's multiple-range test.

RESULTS AND DISCUSSION

Effect of diacetyl on control of *E. coli* O157:H7 and *Salmonella* Typhimurium mixed with *P. acidilactici* in a laboratory medium. Figure 1a shows the growth (CFU/ml) of *E. coli* O157:H7 in the laboratory medium containing *P. acidilactici.* In LBHI medium with only *P. acidilac-*

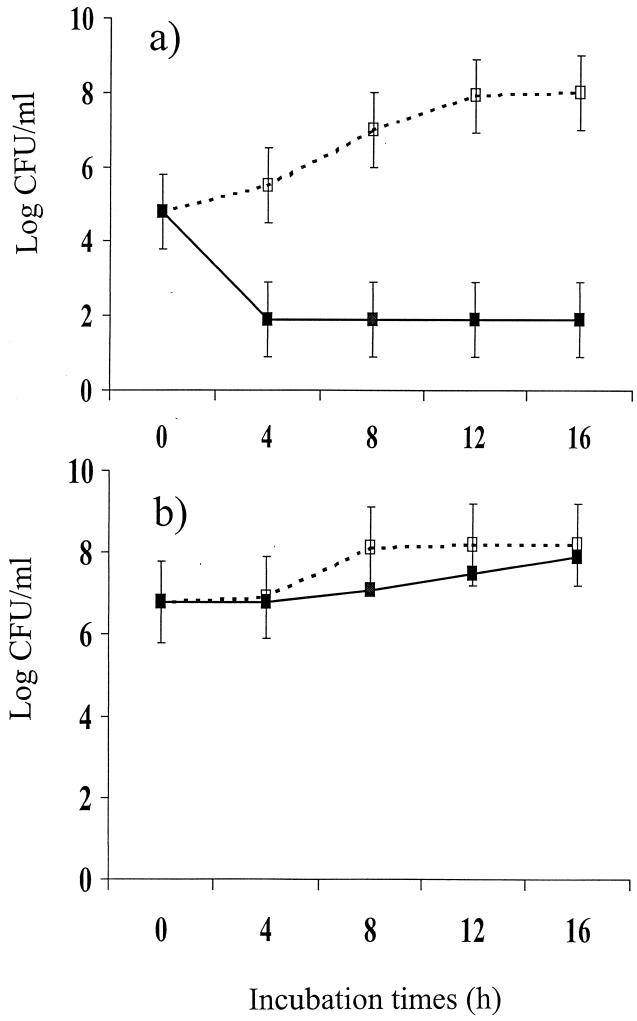

FIGURE 2. *Effect of diacetyl on the growth of mixed cultures of* Salmonella *Typhimurium (a) and* P. acidilactici *(b) in a laboratory medium with (■) or without 50 ppm diacetyl (□).*

tici, the numbers of *E. coli* O157:H7 increased from 4.30 to 7.50 log CFU/ml during incubation for 16 h at 40°C. However, the numbers of *E. coli* O157:H7 in LBHID with *P. acidilactici* decreased to under 2.00 log CFU/ml after 4 h incubation. After that, *E. coli* O157:H7 grew slowly to 3.50 log CFU/ml over 8 to 16 h incubation. The difference in the growth of *E. coli* O157:H7 between LBHI and LBHID was significant ($P < 0.05$). With 50 ppm diacetyl, the numbers of *E. coli* O157:H7 were significantly inhibited ($P < 0.05$). Figure 1b shows that the growth of *P. acidilactici* was not affected significantly ($P > 0.05$) by 50 ppm of diacetyl.

In a mixed culture of *Salmonella* Typhimurium and *P. acidilactici* in the laboratory medium, diacetyl also strongly ($P < 0.05$) inhibited the growth of *Salmonella* Typhimurium (Fig. 2a). The decrease was about 6.00 log CFU/ml in LBHID after 16 h incubation. However, the growth of *P. acidilactici* was not affected significantly by the addition of 50 ppm of diacetyl ($P > 0.05$) after 16 h incubation (Fig. 2b). With higher concentrations of diacetyl (>50 ppm), *E. coli* O157:H7 and *Salmonella* Typhimurium numbers decreased more rapidly during incubation (data not shown). The final pH of the broth was around 4.3 in both experiments regardless of diacetyl addition, possibly because acid production of the starter culture was not affected. With acidic conditions, diacetyl showed stronger antimicrobial activity than at a relatively high pH (>7.0) *(13).* In laboratory experiments, we found that diacetyl can be used as an effective antimicrobial agent active against *E. coli* O157:H7 and *Salmonella* Typhimurium in the presence of *P. acidilactici.*

Effect of diacetyl on control of *E. coli* O157:H7 and *Salmonella* Typhimurium with *P. acidilactici* during salami fermentation. After 24 h fermentation, the pH of the salami with *E. coli* O157:H7 and *P. acidilactici* was reduced from 5.80 to 4.78 without diacetyl (Table 1). The addition of 50, 100, 200, and 300 ppm of diacetyl had no significant effect on final pH, compared to the control ($P > 0.05$), which suggested that 50 to 300 ppm of diacetyl did not affect the acid production of *P. acidilactici* during meat fermentation. Without other spices and ingredients, *E. coli* O157:H7 grew in the presence of starter culture during salami fermentation. Initial counts of *E. coli* O157:H7 increased from 7.00 to 7.60 log CFU/g after 24 h fermentation without diacetyl. However, the numbers of *E. coli* O157:H7 were reduced to 6.63 log CFU/g in the presence of 300 ppm diacetyl (Fig. 3). About a 1-log difference occurred between the numbers of *E. coli* O157:H7 with and without 300 ppm. No significant differences occurred in the control of *E. coli* O157:H7 ($P > 0.05$) between the control and treatment with 50 ppm diacetyl. These results suggest

TABLE 1. *Effect of diacetyl on pH change in a meat system with a mixture of* P. acidilactici *mixed with* E. coli *O157:H7 or* Salmonella *Typhimurium after 24 h meat fermentation*

Doses of diacetyl (ppm)	*E. coli* O157:H7 plus *P. acidilactici* (pH)		*Salmonella* Typhimurium plus *P. acidilactici* (pH)	
	Before fermentation[a]	After 24 h fermentation[b]	Before fermentation[a]	After 24 h fermentation[b]
0	5.80 ± 0.01 A[c]	4.80 ± 0.08 B	5.80 ± 0.03 A	4.48 ± 0.04 B
50	5.80 ± 0.02 A	4.79 ± 0.10 B	5.80 ± 0.01 A	4.47 ± 0.08 B
100	5.80 ± 0.01 A	4.79 ± 0.06 B	5.80 ± 0.04 A	4.47 ± 0.15 B
200	5.80 ± 0.01 A	4.78 ± 0.12 B	5.80 ± 0.01 A	4.48 ± 0.09 B
300	5.80 ± 0.03 A	4.79 ± 0.09 B	5.80 ± 0.02 A	4.47 ± 0.12 B

[a] Data represent means ± standard deviation of three measurements before fermentation.
[b] Data represent means ± standard deviation of three measurements after 24 h fermentation.
[c] Values by different letters are statistically different ($P < 0.05$).

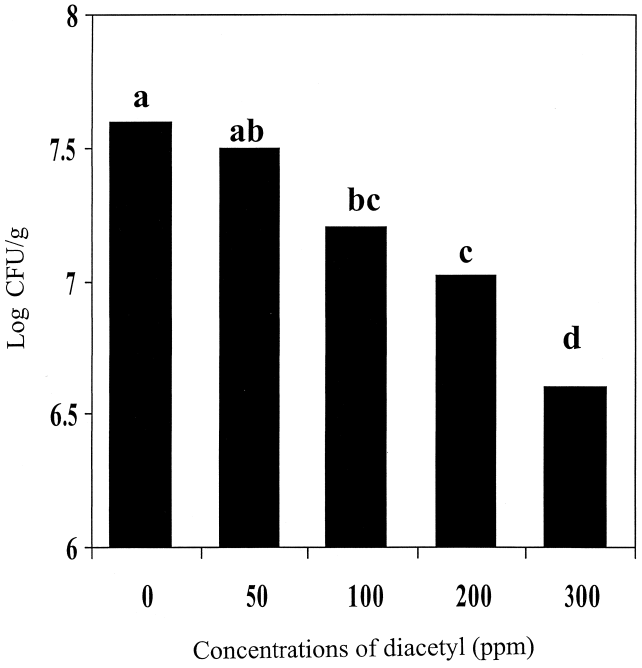

FIGURE 3. *Effect of diacetyl on controlling the growth of* E. coli *O157:H7 during salami meat fermentation. Data with the same superscript are not significantly different (*P > 0.05*).*

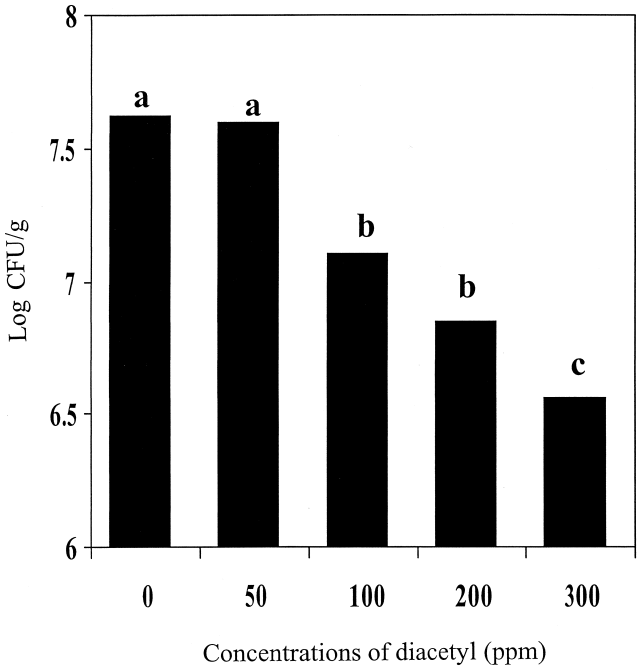

FIGURE 4. *Effect of diacetyl on controlling the growth of* Salmonella *Typhimurium during salami meat fermentation. Data with the same superscript are not significantly different (*P > 0.05*).*

that diacetyl can inhibit the growth of *E. coli* O157:H7 without affecting acid production by *P. acidilactici*. For this experiment, 300 ppm was used for maximum concentration of diacetyl. As a preliminary experiment, we found that high levels of diacetyl (>300 ppm) produced a buttery odor in ground beef (data not shown). The pH results for *Salmonella* Typhimurium plus *P. acidilactici* were similar to those for *E. coli* O157:H7 plus *P. acidilactici* (Table 1). The acid production by *P. acidilactici* mixed with *Salmonella* Typhimurium was not affected by addition of up to 300 ppm diacetyl ($P > 0.05$) (Table 1). The initial inoculum of *Salmonella* Typhimurium was about 6.85 log CFU/g. Without spices and other ingredients, *Salmonella* Typhimurium also increased after 24 h fermentation in the presence of starter culture. The numbers of *Salmonella* Typhimurium mixed with *P. acidilactici* increased from 6.85 log to 7.60 log CFU/g, whereas the numbers were reduced to 6.58 log CFU/g with *P. acidilactici* plus 300 ppm diacetyl. A 1-log difference occurred between the numbers of *Salmonella* Typhimurium with and without 300 ppm of diacetyl. Inhibition of *Salmonella* Typhimurium in the salami system was statistically greater with more than 100 ppm diacetyl ($P < 0.05$).

The effectiveness of diacetyl was much stronger in laboratory medium than in the actual fermented meat. It would be expected that there would be differences between the liquid medium and the solid matrix of the fermented meat. It is possible that there is less homogeneous mixing in the solid matrix and therefore potentially less exposure to compounds added. In addition, there are also potential microenvironmental conditions in the fermented meat that may prevent complete exposure to the full dose of a compound because of diffusion limitations. Also, the fat content of meat should be of concern. In preliminary experiments, we evaluated ground beef with 20% and 25% fat contents to ascertain the effect of fat on the antimicrobial effect of diacetyl. The antimicrobial activity of diacetyl (300 ppm) was significantly decreased with high amounts of fat (>20%) (data not shown). Fat content is the important ingredient for the antimicrobial activity of diacetyl in a meat. We assume that high fat contents will block the antimicrobial activity of diacetyl.

In both the laboratory medium and fermented meat, *P. acidilactici* had more resistance against diacetyl than *E. coli* O157:H7 and *Salmonella* Typhimurium. Jay (*13*) also reported that lactic acid bacteria have more resistance against diacetyl than gram-negative bacteria. Several kinds of spices (e.g., garlic, pepper, sage) reduce foodborne pathogens in fermented meat (*7*). Therefore, the combination of diacetyl and spices could be applied to control foodborne pathogens in salami. Furthermore, pH is also important for diacetyl activity. Manganese ion and oxyrase can stimulate the growth and acid production of meat starter culture (*14*). With stimulators, starter cultures produce acid rapidly. In this environment, diacetyl could more effectively control foodborne pathogens. This research demonstrates the possibilities for using diacetyl as a food additive to control foodborne pathogens such as *E. coli* O157:H7 and *Salmonella* Typhimurium during meat fermentation.

ACKNOWLEDGMENTS

This paper is based upon work supported by the Cooperative State Research, Education, and Extension Service, U.S. Department of Agriculture, under agreement no. 93-34211-8362 and represents contribution no. 99-72-J from the Kansas State Agricultural Experiment Station.

REFERENCES

1. Archer, M. H., V. M. Dillon, G. Campbell-Platt, and J. D. Owens. 1996. Effect of diacetyl on growth rate of *Salmonella typhimurium* determined from detection times measured in a micro-well plate photometer. Food Cont. 7:63–67.
2. Bean, N. H., P. M. Griffin, and C. Ivey. 1990. Foodborne disease outbreaks, 5-year summary, 1983–1987. Morbid. Mortal. Weekly Rep. 39:15–57.
3. Besser, R. E., S. M. Lett, J. T. Weber, M. P. Doyle, T. J. Barrett, J. G. Wells, and P. M. Griffin. 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed cider. JAMA 269:2217–2220.
4. Bills, D. D., and S. Kung. 1990. Biotechnology and food safety. Butterworth-Heinemann, Inc., Stoneham, Mass.
5. Centers for Disease Control and Prevention. 1995. *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami—Washington and California. Morbid. Mortal. Weekly Rep. 44: 157–159.
6. Centers for Disease Control and Prevention. 1996. Surveillance for foodborne-disease outbreaks—USA. 1988–1992, vol. 45, no. SS-5.
7. Ceylan, E., D. H. Kang, and D. Y. C. Fung. 1998. Reduction of *Escherichia coli* O157:H7 by selected spices. IFT annual meeting, Atlanta, Ga.
8. El-Khateib, T., and H. A. El-Rahman. 1987. Effect of garlic and *Lactobacillus plantarum* on growth of *Salmonella typhimurium* in Egyptian fresh sausage and beefburger. J. Food Prot. 50:310–311.
9. Fung, D. Y. C., R. Phebus, D. H. Kang, and C. L. Kastner. 1995. Effect of alcohol-flaming on meat cutting knives. J. Rapid Meth. Automat. Microbiol. 3:237–243.
10. Gupta, K. G., L. Chandiok, and L. Bhatnagar. 1973. Antibacterial activity of diacetyl and its influence on the keeping quality of milk. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B 158:202–205.
11. Hargrove, R. E., F. E. McDonough, and W. A. Mattingly. 1969. Factors affecting survival of *Salmonella* in Cheddar and Colby cheese. J. Milk Food Technol. 32:480–484.
12. Humphrey, T. J. 1994. Contamination of egg shell and contents with *Salmonella enteritidis*: a review. Int. J. Food Microbiol. 21:31–40.
13. Jay, J. 1982. Antimicrobial properties of diacetyl. Appl. Environ. Microbiol. 44:525–532.
14. Kang, D. H., and D. Y. C. Fung. 1997. Effect of manganese ion and oxyrase on control of foodborne pathogens by *Pediococcus acidilactici*. Proceedings of Food Safety Consortium Annual meeting, Kansas City, Mo.
15. Kulshrestha, D. C., and E. H. Marth. 1970. Inhibition of lactic streptococci and some pathogenic bacteria by certain milk-associated volatile compounds as measured by the disc assay. J. Milk Food Technol. 33:305–310.
16. Kulshrestha, D. C., and E. H. Marth. 1974. Inhibition of bacteria by some volatile and nonvolatile compounds associated with milk. *Escherichia coli*. J. Milk Food Technol. 37:510–516.
17. Padhye, N. V., and M. P. Doyle. 1992. *Escherichia coli* O157:H7: epidemiology, pathogenesis, and methods for detection in food. J. Food Prot. 55:555–565.
18. SAS. 1991. SAS/STAT users guide, release 6.03. SAS Institute Inc., Cary, N.C.
19. Tate, C. R., R. G. Miller, and E. T. Mallinson. 1992. Evaluation of two isolation and two nonisolation methods for detecting naturally occurring salmonellae from broiler flock environmental drag-swab samples. J. Food Prot. 55:964–967.
20. Thomas, L. V., and J. Wimpenny. 1996. Competition between *Salmonella* and *Pseudomonas* species growing in and on agar, as affected by pH, sodium chloride concentration and temperature. Int. J. Food Microbiol. 29:361–370.
21. Van Neil, C. B., A. J. Kluyver, and H. G. Derx. 1929. Uber das Butteraroma. Biochem. Z. 210:234–251.
22. Zhao, T., and M. P. Doyle. 1994. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in commercial mayonnaise. J. Food Prot. 57: 780–783.