SUPPLEMENT

Influence of pH Treatments on Survival of *Escherichia coli* O157:H7 in Continuous Cultures of Rumen Contents

BRANDOLYN H. THRAN,* HUSSEIN S. HUSSEIN,*,[1] DOUG REDELMAN,† AND GEORGE C.J. FERNANDEZ‡

Department of Animal Biotechnology, †Cytometry Center, and ‡Nevada Agricultural Experiment Station, University of Nevada, Reno, Nevada 89557

The pH (i.e., 5.5, 5.75, 6.0, 6.25, 6.5, 6.75, 7.0, and 7.25) effect on *Escherichia coli* O157:H7 in an artificial rumen model was investigated. Eight fermenters were inoculated with bovine rumen fluid and were supplied with a diet (75 g of dry matter daily in 12 equal portions [every 2 hr]) containing similar forage-to-concentrate ratio. After an adaptation period (i.e., 3 days for adjusting the rumen fluid [pH 6.2] microbial population to the test pH and 4 days for adjustment to the diet at the test pH), each fermenter was inoculated with 10^9 cells of *E. coli* O157:H7. Samples were collected hourly for 12 hr and every 2 hr for an additional 12 hr and were analyzed by flow cytometer. *E. coli* O157:H7 could not be quantified after 24 hr, and detection was only possible after enrichment. Because the pathogen could not be detected 5 days postinoculation (i.e., Day 13), the fermenters were reinoculated with *E. coli* O157:H7 on Days 17 and 22. *E. coli* O157:H7 numbers decreased from 10^6 to 10^4/ml of fermenter contents in a quadratic ($P < 0.05$) fashion over the 24-hr sampling period, and the rate of reduction was slower ($P < 0.05$) for pH 7.0 than for other pH treatments. Results suggested that *E. coli* O157:H7 population were decreased by competitive exclusion and were not affected by culture pH. Exp Biol Med 228:365–369, 2003

Key words: *Escherichia coli*; cattle; continuous culture; food-borne pathogens

This work was supported by the U.S. Department of Agriculture Integrated Research, Education, and Extension Competitive Grants Program Grant 2001-05062. This research was also supported in part by Nevada Agricultural Experiment Station (publication no. 5302450).

[1] To whom request for reprints should be addressed at Animal Biotechnology, University of Nevada, Mail Stop 202, Reno, NV 89557. E-mail: hhussein@agnt1.ag.unr.edu

1535-3702/03/2284-0365$15.00

Escherichia coli O157:H7 has been associated with numerous worldwide outbreaks of human food-borne illnesses (1–3). Because studies have directly linked several human illness outbreaks to consumption of undercooked ground beef or other beef products, cattle are considered reservoirs of this pathogen (4–7). At slaughter, contamination of beef with *E. coli* O157:H7 usually occurs from feces on the hide or from the digesta released by nicking the gastrointestinal tract. Because contamination is confined to the carcass surface (8), efforts have been devoted to develop and implement postharvest control methods to reduce *E. coli* O157:H7 contamination. These efforts resulted in developing and implementing the Hazard Analysis and Critical Control Point system to assure safety of beef and other meat products (9). The postharvest control methods and their efficacy have been reviewed recently (10). These methods include trimming and washing (11, 12), spraying with sanitizers (13–15), hot water washing (16, 17), using dips (18, 19), using food additives (20), and irradiation (21, 22).

Recently, attention has been given to development and evaluation of preharvest strategies that would minimize or reduce *E. coli* O157:H7 contamination before sending cattle to slaughter. Implementation of control measures at the farm level combined with effective postharvest measures would improve beef safety and reduce consumers' concern. Several on-farm factors have been found to interact simultaneously and affect carriage and shedding of *E. coli* O157:H7 by cattle. These include animal factors (23), manure handling (24), drinking water (25), using feed additives (26), feeding probiotics (26), feeding management (27), and dietary ingredients (28–32).

Diet composition (33) has been shown to significantly alter survival, proliferation, and shedding of *E. coli* O157:H7 by sheep (28, 34) and cattle (32). However, the modes and sites (rumen, small intestine, or large intestine)

of such actions are not identified. Recently, the diet effects on ruminal proliferation and fecal shedding of *E. coli* O157:H7 in inoculated cattle were investigated (26, 33). Regardless of the various daily rumen pH ranges (i.e., 5.6 to 6.6, 5.9 to 7.0, and 5.9 to 6.8) created by feeding different concentrates (corn, cottonseed/barley, or barley, respectively), *E. coli* O157:H7 was quickly eliminated from the rumen but persisted in the feces for 67 days (33). Similar responses were observed when high-concentrate or high-forage diets were fed (26). Therefore, it was suggested that rumen pH may have less effect on survival and proliferation of *E. coli* O157:H7. In these studies (26, 33), a limited number of rumen pH treatments (created by the diets fed) were tested and the response may have been masked by animal variations. Therefore, this study was designed to assess survival and proliferation of *E. coli* O157:H7 under rumen pH treatments of practical implication. To achieve this, eight pH treatments were selected to cover the wide range of ruminal pH under normal feeding conditions (34), which vary from high concentrate (in the feedlot) to high or all forage (under grazing conditions). The pH treatments were tested by using a dual-flow continuous culture fermenter system that mimics ruminal microbial fermentation while minimizing variations.

Materials and Methods

Animals and Collection of Rumen Fluid. Two ruminally cannulated mature Angus steers were used as donors of rumen fluid to inoculate the dual-flow continuous culture fermenter system. The steers were gradually (over a 1-month period) adapted to a diet containing 50% forage (grass hay) and 50% concentrate (corn) on a dry matter basis and had *ad libitum* access to this diet for 2 weeks before collection of rumen fluid. The diet was formulated to meet or exceed nutrient requirements of the steers (35). The rumen fluid was collected (by using a vacuum pump) from each steer approximately 2 hr postfeeding and was strained immediately through four layers of cheesecloth into a pre-warmed, insulated thermos.

Continuous Culture System and Operation. The dual-flow continuous culture fermenter system was developed (36) and modified (37) to simulate differential solid-liquid removal rates occurring in the rumen environment. Evaluation and validation of the efficacy of this system in simulating ruminal fermentation in cattle (37, 38) and sheep (39, 40) were documented. The system at the University of Nevada-Reno consists of eight fermenters of 1020 ml of working volume each. Upon arrival to the laboratory, the rumen fluids from both steers were combined (on an equal volume basis) and used to inoculate the fermenters. Each fermenter was supplied daily with 75 g of dry matter of the ground (2-mm screen) diet (13.3% protein; containing, on dry matter basis, 49.6% grass hay, 35.8% corn, 11.9% soybean meal, 1.59% limestone, 0.67% Na_2SO_4, and 0.42% mineral/vitamin mix) by an automated feeding mechanism adjusted to deliver the diet in 12 equal portions (every 2 hr) over a 24-hr period to establish steady-state conditions. Each fermenter was continuously infused with a mineral buffer (artificial saliva) solution (41) containing urea (0.5 g/l) at a rate of 1.8 ml/min to obtain a liquid dilution rate of 10% hr^{-1}. Solid (overflow) dilution rate was maintained at 5% hr^{-1} by removing liquid through a filter at 0.9 ml/min. Anaerobic conditions were achieved by continuous infusion of N_2 at a rate of 40 ml/min. Maintaining the fermenters' temperature at 39°C and mixing of their contents were achieved by using the VirTis Omni-Culture fermenter base units (The VirTis Company, Gardiner, NY). The test pH of each fermenter (i.e., 5.5, 5.75, 6.0, 6.25, 6.5, 6.75, 7.0, and 7.25 ± 0.05) was maintained by automated infusion of 3 *N* HCl or 5 *N* NaOH regulated by a pH controller (Cole-Parmer, Vernon Hills, IL).

Experimental Design. The experimental design was a randomized complete block design (42) and consisted of three inoculation periods (blocks). The eight pH treatments were allocated at random to the eight fermenters. At the beginning of the experiment, the first 3 days were used for gradual adjustment of the microbial population in the rumen fluid (pH 6.2) inoculum to the test pH. The following 4 days were used for adjustment of the microbial population to the diet at the test pH. On Day 8, each fermenter was inoculated with 1 ml of *E. coli* O157:H7 (ATCC 43888) suspension (10^9 cells/ml). On Day 13 (5 days postinoculation), no *E. coli* O157:H7 was found in the fermenters even after enrichment (43). Therefore, inoculation of the fermenters with *E. coli* O157:H7 was repeated on Days 17 and 22 to allow for three replications for each pH treatment. No carry over effect was expected because on Day 5 of each inoculation, no *E. coli* O157:H7 was detected in the fermenter contents before or after enrichment.

***E. coli* O157:H7 Inoculum.** The *E. coli* O157:H7 inoculum was prepared by growing the cells in tryptic soy broth at 37°C for 10 hr and then its concentration was estimated spectrophotometrically (OD_{600}) based on previously established growth curves. *E. coli* O157:H7 cells were concentrated by centrifugation at 3000*g* for 10 min and resuspended in sterile saline at approximately 10^9 cells/ml. Actual starting concentrations were confirmed by plating serial dilutions of the inoculum on tryptic soy agar.

Sample Collection. Samples (1 ml each) were collected from each fermenter 1 hr before and every hour for 12 hr postinoculation. Samples then were collected every 2 hr for an additional 12 hr. Samples were immediately placed on ice and stored at 4°C. At the end of the 24-hr sampling period, all samples were analyzed to detect and quantify *E. coli* O157:H7 by using a flow cytometric method (43).

Sample Analysis. From each sample, a 100-μl aliquot was taken and labeled with 100 μg of fluorescein (FITC)-labeled affinity purified antibody to *E. coli* O157:H7 (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Positive controls were pure cultures of *E. coli* O157:H7 (ATCC 43888). The measurements were performed with a four-color XL-MCL flow cytometer with

System II software (Beckman Coulter, Fullerton, CA). The instrument was operated at the high flow rate that examines samples at approximately 40 μl/min. Because the samples contained large numbers of extraneous particles (i.e., feed or other bacterial species), the instrument was normally thresholded on green fluorescence to collect relatively larger numbers of the specifically labeled *E. coli* O157:H7 (43). Data files were collected for standard times and were used in combination with the known flow rates to calculate approximate cell (i.e., *E. coli* O157:H7) concentrations.

Statistical Analysis. The starting concentrations of *E. coli* O157:H7 for the first, second, and third inoculations were confirmed by plate counts to be 1.3×10^{10}, 2.9×10^{10}, and 3.9×10^{9} cells/ml of inoculum (i.e., 1.3×10^{7}, 2.9×10^{7}, and 3.9×10^{6} cells/ml of fermenter contents), respectively. Because of these variations in the starting concentrations, the numbers of *E. coli* O157:H7 in every fermenter at each sampling time (measured by flow cytometry) were standardized and expressed as percentages of the initial dose. These percentages of *E. coli* O157:H7 remaining in the fermenters at a given time were analyzed as a repeated measure in a randomized complete block design with a factorial (pH × time) treatment structure by using Proc MIXED of SAS (44). The covariance structures (ar[1], cs, and toeplitz) among the different sampling times were compared by using the type option in SAS Proc MIXED (44). The ar(1) covariance structure was selected to model the correlations among the sampling times based on the smallest AIC value (44). The sampling time (number of hours postinoculation) was treated as quantitative, and the significance ($P < 0.05$) of the linear, quadratic, linear × pH interaction, and quadratic × pH interaction were tested. The percentage of *E. coli* O157:H7 remaining at any given time for each pH treatment was estimated from the linear and quadratic coefficients of the quadratic regression model included in the MIXED model. Significant differences ($P < 0.05$) among linear or quadratic coefficients for the various pH treatments were tested by using *t* test in the Proc MIXED solution option (44). At 12 hr postinoculation, the percentages of *E. coli* O157:H7 remaining in the continuous culture system for the pH tested were compared by using the least-squares means option in Proc MIXED (44). At 24 hr postinoculation, the percentage of *E. coli* O157:H7 remaining were also compared in a similar fashion.

Results

Preinoculation (1 hr before) samples from each of the eight fermenters were negative for *E. coli* O157:H7 when tested directly or after enrichment. After inoculation, the actual numbers of *E. coli* O157:H7 (data not shown) decreased immediately for all pH treatments except for pH 7.0. At this pH, *E. coli* O157:H7 numbers increased for 4 hr before starting to decrease in a fashion similar to that for the remaining pH treatments.

The regression lines depicting the decrease in *E. coli* O157:H7 numbers as a percentage of initial dose are illustrated in Figure 1. Each line represents the fitting of data collected from the three inoculations (i.e., three replications) for each pH treatment. The decrease in *E. coli* O157:H7 numbers was quadratic ($P < 0.05$) for all pH treatments. This is confirmed by the coefficients of the quadratic effects presented in Table I. Based on the data in Figure 1, the numbers of *E. coli* O157:H7 remaining in the fermenters decreased at a slower rate for the culture at pH 7.0 when compared with those at other pH treatments. This observation is also confirmed by the magnitude of the linear and quadratic coefficients of the quadratic model (Table I).

A preliminary experiment was conducted to assess the effects of the above mentioned pH treatments on the same *E. coli* O157:H7 (at the same inoculation dose) used in the current study. In this preliminary experiment, samples were collected every hour and analyzed by flow cytometer. Results (data not shown) revealed that *E. coli* O157:H7 numbers decreased rapidly during the first 12 hr postinoculation and then decreased at a slower rate for the following 12 hr. At 24 hr postinoculation, the number of *E. coli* O157:H7 remaining in the fermenters fell below the sensitive range (i.e., $<10^{4}$ cells/ml) of *E. coli* O157:H7 quantification (43). Therefore, it was decided to terminate the sampling at 24 hr postinoculation.

Regardless of the pH treatment, the rates of reduction of *E. coli* O157:H7 numbers in the fermenters were fast during the first 12 hr postinoculation. Therefore, we attempted to estimate the percentages of *E. coli* O157:H7 numbers remaining in the fermenters at 12 hr postinoculation for each of the pH treatments by using the quadratic regression model (i.e., $Y = \beta_0 - \beta_1 t + \beta_2 t^2$, where Y is the percentage of remaining *E. coli* O157:H7 numbers at *t* incubation time, β_0 is the Y intercept, and both β_1 and β_2 are

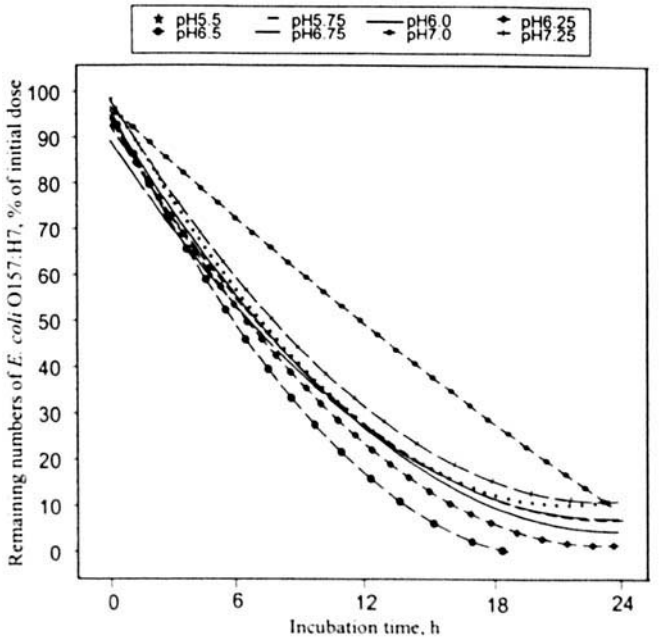

Figure 1. Remaining numbers of *E. coli* O157:H7 (expressed as a percentage of the initial dose) in continuous cultures of rumen contents as influenced by culture pH.

Table I. Comparisons Among the Intercepts (β_0) and Each of the Linear (β_1) and Quadratic (β_2) Sets of Coefficients (at a given time [t]) of the Quadratic Regression Model[a] Used to Examine the Effect of pH on the Remaining Number of *E. coli* O157:H7 (as a percentage of initial dose) Over Time in Continuous Cultures of Rumen Contents

Regression coefficients	pH treatments							
	5.5	5.75	6.0	6.25	6.5	6.75	7.0	7.25
β_0[b]	99.8	96.0	101.8	95.8	98.1	94.4	103.7	99.6
β_1	8.38^{cd}	8.39^{cd}	8.59^{c}	8.92^{c}	10.17^{c}	8.03^{cd}	4.84^{d}	8.33^{cd}
β_2	0.18^{c}	0.19^{c}	0.19^{c}	0.20^{c}	0.26^{c}	0.18^{c}	0.02^{d}	0.19^{c}

[a] $Y = \beta_0 - \beta_1 t + \beta_2 t^2$ where Y is the percentage of remaining number of *E. coli* O157:H7 at t incubation time.
[b] Intercept values were not different ($P > 0.05$).
[c] and [d] Coefficients in the same row with uncommon superscripts differ ($P < 0.05$).

the linear and quadratic coefficients for a specific pH treatment). The estimated percentages (data not shown) were then compared for each two pH treatments at 12 hr postinoculation. The difference (percentage units) between the estimated values for each set of two pH treatments and the significance of such difference are presented in Table II. At 12 hr postinoculation (Table II), only the effect of pH 7.0 was different ($P < 0.05$) from those of other pH treatments that were all similar ($P > 0.05$). This observation suggests that regardless of culture pH (except for pH 7.0), *E. coli* O157:H7 numbers decreased at a fast rate. Although the decrease in *E. coli* O157:H7 numbers at pH 7.0 during the first 12 hr postinoculation was slower than that for other pH treatments, the final levels of number reduction at 24 hr postinoculation were not different ($P > 0.05$; data not shown). This could be explained by the fact that rate of number reduction was faster at pH 7.0 during the second 12 hr postinoculation, whereas the rates of reduction were slower for the other pH treatments during that time.

Discussion

The rumen environment is very complex and has evolved over many years to allow for survival and proliferation of a specific anaerobic microbial population that supports survival and production of the host animal through fermentation (45). Therefore, it is difficult for a transient organism to survive in this highly competitive environment. Within the rumen, there are many inhibitory factors (e.g., rumen pH and fermentation end-products) that limit growth of transient bacterial species.

Table II. Comparisons Among Estimated Percentages of *E. coli* O157:H7 Remaining in Continuous Cultures of Rumen Contents for the pH Tested at 12-hr Postinoculation[a]

pH	5.5	5.75	6.0	6.25	6.5	6.75	7.0	7.25
5.5		2.0	0	6.9	12.6	1.6	24.1[b]	2.2
5.75			2.3	4.9	10.7	0	26.0[b]	4.1
6.0				7.2	13.0	2.0	23.7[b]	1.9
6.25					5.7	5.2	31.0[b]	9.1
6.5						10.9	36.7[b]	14.8
6.75							25.7[b]	3.9
7.0								21.8[b]
7.25								

[a] Each number listed reflects the difference between the percentages of initial dose of *E. coli* O157:H7 remaining at 12-hr postinoculation for the compared pH treatments.
[b] The difference between the estimated percentage remaining for each two pH treatments was significant ($P < 0.05$).

The observation that *E. coli* O157:H7 could not proliferate and decreased in numbers within 24 hr in the continuous culture system at the wide pH range (i.e., 5.5–7.25) tested (Fig. 1) is in agreement with results from studies investigating survival of *E. coli* O157:H7 *in vivo* (26, 33). Buchko *et al.* (33) and Tkalcic *et al.* (26) reported that *E. coli* O157:H7 was rapidly removed from cattle with rumens of varying pH (i.e., ranging from 5.6 to 7.0).

Because fermentation of different diets results in various profiles of acid end-products that alter rumen pH, it was suggested (28, 30, 32) that dietary manipulation may affect survival and proliferation of *E. coli* O157:H7 in the rumen environment. However, our results (Fig. 1) suggest that competitive exclusion by the rumen microorganisms most probably decreased the numbers of *E. coli* O157:H7 within 24 hr from 10^6 and 10^7 to 10^4 and 10^5 cells/ml of artificial rumen contents, regardless of culture pH. It appears that the rumen pH, as a function of fermentation of dietary components, is less effective in decreasing the numbers of *E. coli* O157:H7 entering cattle rumens. Therefore, our data and those of others (26, 33) suggest that the diet effects on *E. coli* O157:H7 entering the gastrointestinal tract of cattle is postruminally through specific ingredients or components. Diet effects on the small or large intestinal environments may be through altering colonization, proliferation, and the subsequent shedding of this food-borne pathogen.

1. Tarr PI. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin Infect Dis **20**:1–10. 1994.
2. Armstrong GL, Hollingsworth J, Morris JG Jr. Emerging food-borne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. Epidemiol Rev **18**:29–51. 1996.
3. Buchanan RL, Doyle MP. Food-borne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli*. Food Technol **51**:69–76. 1997.
4. Riley LW, Remis RS, Helgerson SD, McGee HB, Well JG, Davis BR,

Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, Blake PA, Cohen ML. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med **308**:681–685, 1983.
5. Bell BP, Goldoft M, Griffin PM, Davis MA, Gordon DC, Tarr PI, Bartleson CA, Lewis JH, Barrett TJ, Wells JG, Baron R, Kobayashi J. A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. J Am Med Assoc **272**:1349–1353, 1994.
6. Hancock DD, Besser TE, Kinsel ML, Tarr PI, Rice DH, Paros MG. The prevalence of *Escherichia coli* O157:H7 in dairy and beef cattle in Washington state. Epidemiol Infect **113**:199–207, 1994.
7. Hussein HS, Thran BH, Hall MR. *Escherichia coli* O157:H7 as a food-borne pathogen: microbial, environmental, and nutritional factors affecting prevalence in ruminants and outbreaks in humans. Environ Nutr Interact **3**:277–311, 1999.
8. Dickson JS, Siragusa GR. Survival of *Salmonella typhimurium*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* during storage on beef sanitized with organic acids. J Food Safety **14**:313–327, 1994.
9. Karr KJ, Maretzki AN, Knabel S. Meat and poultry companies assess USDA's hazard analysis and critical control point system. Food Technol **48**:117–122, 1994.
10. Hussein HS, Lake SL, Ringkob TP. Cattle as a reservoir of Shiga-like toxin-producing *Escherichia coli* including O157:H7-pre- and post-harvest control measures to assure beef safety. Prof Anim Sci **17**:1–16, 2001.
11. Hardin MD, Acuff GR, Lucia LM, Oman JS, Savell JW. Comparison of methods for decontamination from beef carcass surfaces. J Food Prot **58**:368–374, 1995.
12. Reagan JO, Acuff GR, Buege DR, Buyck MJ, Dickson JS, Kastner CL, Marsden JL, Morgan JB, Nickelson R II, Smith GC, Sofos JN. Trimming and washing of beef carcasses as a method for improving the microbiological quality of meat. J Food Prot **59**:751–756, 1996.
13. Brackett RE, Hao YY, Doyle M. Ineffectiveness of hot acid sprays to decontaminate *Escherichia coli* O157:H7 on beef. J Food Prot **57**:198–203, 1994.
14. Podolak RK, Zayas JF, Kastner C, Fung DYC. Inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7 on beef by application of organic acids. J Food Prot **59**:370–373, 1996.
15. Jordan SL, Glover J, Malcolm L, Thomson-Carter FM, Booth IR, Park SF. Augmentation of killing of *Escherichia coli* O157:H7 by combinations of lactate, ethanol, and low pH conditions. Appl Environ Microbiol **65**:1308–1311, 1999.
16. Barkate ML, Acuff GR, Lucia LM, Hale DS. Hot water decontamination of beef carcasses for reduction of initial bacterial numbers. Meat Sci **35**:397–401, 1993.
17. Dorsa WJ, Cutter CN, Siragusa GR. Effectiveness of a steam-vacuum sanitizer for reducing *Escherichia coli* O157:H7 inoculated to beef carcass surface tissue. Lett Appl Microbiol **23**:61–63, 1996.
18. Stevens KA, Sheldon BW, Klapes NA, Klaenhammer TR. Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. Appl Environ Microbiol **57**:3613–3615, 1991.
19. Hathcox AK, Beuchat LR. Inhibitory effects of sucrose fatty acid esters, alone and in combination with ethylenediaminetetraacetic acid and other organic acids, on viability of *Escherichia coli* O157:H7. Food Microbiol **13**:213–225, 1996.
20. Kang DH, Fung DYC. Effect of diacetyl on controlling *Escherichia coli* O157:H7 and *Salmonella typhimurium* in presence of started culture in a laboratory medium and during meat fermentation. J Food Prot **62**:975–979, 1999.
21. Thayer DW, Boyd G. Elimination of *Escherichia coli* O157:H7 in meats by γ-irradiation. Appl Environ Microbiol **59**:1030–1034, 1993.
22. Wood OB, Bruhn CM. Position of the American Dietetic Association: food irradiation. J Am Diet Assoc **100**:246–253, 2000.
23. Herriott DE, Hancock DD, Edel ED, Carpenter LV, Ric DH, Besser TH. Association of herd management factors with colonization of dairy cattle by Shiga toxin-positive *Escherichia coli* O157:H7. J Food Prot **61**:802–807, 1998.
24. Hancock DD, Besser T, Rice D, Garber LP, Wells SJ, Harriot DE, Ebel ED. Identification of management factors influencing on-farm prevalence of *Escherichia coli* O157:H7. PB1-PB4. Proc 8th Int Congr of Anim Hyg, Budapest, Hungary, 1994.
25. Hancock DD, Besser TE, Rice DH. Ecology of *Escherichia coli* O157:H7 in cattle and impact of management practices. In: Kaper JB, O'Brien AB, Eds. *Escherichia coli* O157:H7 and Other *Shiga* Toxin-Producing *E. coli* Strains. Washington, DC: American Society for Microbiology, pp85–91, 1998.
26. Tkalcic S, Brown CA, Harmon BG, Jain AV, Mueller EPO, Parks A, Jacobsen KL, Martin SA, Zhao T, Doyle MP. Effects of diet on rumen proliferation and fecal shedding of *Escherichia coli* O157:H7 in calves. J Food Prot **63**:1630–1636, 2000.
27. Jordan D, McEwen SA. Effect of duration of fasting and a short-term high-roughage ration on the concentration of *Escherichia coli* Biotype 1 in cattle feces. J Food Prot **61**:531–534, 1998.
28. Kudva IT, Hatfield PG, Hovde CJ. Effect of diet on the shedding of *Escherichia coli* O157:H7 in a sheep model. Appl Environ Microbiol **61**:1363–1370, 1995.
29. Dargatz DA, Wells SJ, Thomas LA, Hancock DD, Garber LP. Factors associated with the presence of *Escherichia coli* O157 in feces of feedlot cattle. J Food Prot **60**:466–470, 1997.
30. Kudva IT, Hunt CW, Williams CJ, Nance UM, Hovde CJ. Evaluation of dietary influences on *Escherichia coli* O157:H7 shedding by sheep. Appl Environ Microbiol **63**:3878–3886, 1997.
31. Lynn TV, Hancock DD, Besser TE, Harrison JH, Rice DH, Stewart NT, Rowan LL. The occurrence and replication of *Escherichia coli* in cattle feeds. J Dairy Sci **81**:1102–1108, 1998.
32. Hovde CJ, Austin PR, Cloud KA, Williams CJ, Hunt CW. Effect of cattle diet on *Escherichia coli* O157:H7 acid resistance. Appl Environ Microbiol **65**:3233–3235, 1999.
33. Buchko SL, Holley RA, Olson WO, Gannon VPJ, Veira DM. The effect of different grain diets on fecal shedding of *Escherichia coli* O157:H7 by steers. J Food Prot **63**:1467–1474, 2000.
34. Owens FN, Goetsch AL. Ruminal fermentation. In: Church DC, Ed. The Ruminant Animal, Digestive Physiology and Nutrition. Prospect Heights, IL: Waveland Press, pp145–171, 1988.
35. NRC. Nutrient Requirements of Beef Cattle, 7th revised ed. Washington, DC: National Academy Press, 1996.
36. Hoover WH, Crooker BA, Sniffen CJ. Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen contents. J Anim Sci **43**:528–534, 1976.
37. Hannah SM, Stern MD, Ehle FR. Evaluation of a dual flow continuous culture system for estimating bacterial fermentation in vivo of mixed diets containing various soybean products. Anim Feed Sci Technol **16**:51–62, 1986.
38. Hannah SM, Stern MD. Effect of supplemental niacin or niacinamide and soybean source on ruminal bacterial fermentation in continuous culture. J Anim Sci **61**:1253–1263, 1985.
39. Hussein HS, Jordan RM, Stern MD. Ruminal protein metabolism and intestinal amino acid utilization as affected by dietary protein and carbohydrate sources in sheep. J Anim Sci **69**:2134–2146, 1991.
40. Hussein HS, Stern MD, Jordan RM. Influence of dietary protein and carbohydrate sources on nitrogen metabolism and carbohydrate fermentation by ruminal microbes in continuous culture. J Anim Sci **69**:2123–2133, 1991.
41. Weller RA, Pilgrim AF. Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. Br J Nutr **32**:341–351, 1974.
42. Steel RGD, Torrie JH, Dickey DA. Principles and Procedures of Statistics: A Biometrical Approach. New York: McGraw-Hill Publishing, 1997.
43. Hussein HS, Thran BH, Redelman D. Detection of *Escherichia coli* O157:H7 in bovine rumen fluid and feces by flow cytometry. Food Control **13**:387–391, 2002.
44. SAS. Statistical Analysis Systems User's Guide: Statistics, version 8.2. Cary, NC: SAS Institute, 2001.
45. Hungate RE. The Rumen and its Microbes. New York: Academic Press, 1966.