Journal of Food Protection, Vol. 70, No. 3, 2007, Pages 543–550

Effect of Tannins on the In Vitro Growth of *Escherichia coli* O157:H7 and In Vivo Growth of Generic *Escherichia coli* Excreted from Steers

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MS 06-290: Received 25 May 2006/Accepted 5 October 2006

ABSTRACT

The effect of commercially available chestnut and mimosa tannins in vitro (experiment 1) or in vivo (experiment 2) on the growth or recovery of *Escherichia coli* O157:H7 or generic fecal *E. coli* was evaluated. In experiment 1, the mean growth rate of *E. coli* O157:H7, determined via the measurement of optical density at 600 nm during anaerobic culture in tryptic soy broth at 37°C, was reduced ($P < 0.05$) with as little as 400 μg of either tannin extract per ml of culture fluid. The addition of 200, 400, 600, 800, and 1,200 μg of tannins per ml significantly ($P < 0.01$) reduced the specific bacterial growth rate when compared with the nontannin control. The specific growth rate decreased with increasing dose levels up to 800 μg of tannins per ml. Bacterial growth inhibition effects in chestnut tannins were less pronounced than in mimosa tannins. Chestnut tannin extract addition ranged from 0 to 1,200 μg/ml, and a linear effect ($P < 0.05$) was observed in cultures incubated for 6 h against the recovery of viable cells, determined via the plating of each strain onto MacConkey agar, of *E. coli* O157:H7 strains 933 and 86-24, but not against strain 6058. Similar tests with mimosa tannin extract showed a linear effect ($P < 0.05$) against the recovery of *E. coli* O157:H7 strain 933 only. The bactericidal effect observed in cultures incubated for 24 h with the tannin preparations was similar, although it was less than that observed from cultures incubated for 6 h. When chestnut tannins (15 g of tannins per day) were infused intraruminally to steers fed a Bermuda grass hay diet in experiment 2, fecal *E. coli* shedding was lower on days 3 ($P < 0.03$), 12 ($P = 0.08$), and 15 ($P < 0.001$) when compared with animals that were fed a similar diet without tannin supplementation. It was concluded that dietary levels and sources of tannins potentially reduce the shedding of *E. coli* from the gastrointestinal tract.

Plant tannins are natural phenolic compounds that generally consist of two distinct types, hydrolyzable tannins (molecular weight = 500 to 3,000) and condensed tannins (molecular weight = 1,000 to 20,000), although other tannins occur that are combinations of these two basic chemical groups *(13, 22).* The hydrolyzable tannins consist of a core of glucose esterified with gallic and hexahydroxydiphenic acids *(13, 24, 30).* Condensed tannins comprise flavan-3-ol (catechin) units condensed into polymers through C-C bonding *(13, 30).* The two types of tannins differ in their nutritional significance and toxic effects *(19, 29).* Both tannins precipitate protein, but condensed tannins are not readily degraded in the gut, while hydrolyzable tannins undergo microbial and acid hydrolysis with the release of simpler phenolics *(24, 31).*

The antimicrobial activity of tannins has been well reviewed *(6, 13, 30).* Several reports have indicated that green tea (*Camellia sinensis*) condensed tannins show antibacterial or bactericidal activities *(35, 36).* The evidence suggests, however, that the bactericidal activity of green tea tannins is lower against gram-negative bacteria than against gram-positive bacteria *(15),* and this has been attributed to the presence of the lipopolysaccharide cell surface of gram-negative bacteria *(15).* In addition, the growth of ruminal bacteria (*Butyrivibrio fibrisolvens, Ruminobacter amylophilus,* and *Streptococcus bovis*) was reduced by condensed tannins, but a strain of *Prevotella ruminicola* was tolerant to <600 μg of condensed tannins per ml *(16, 20).* Some microbial inhibitions caused by tannins likely result from inhibited nutrient transport of nutrients into the cell that retards growth *(30).* However, the mechanisms by which tannins inhibit pathogenic bacteria have not been clearly elucidated. The objectives of this study were to examine whether different sources of tannins (condensed versus hydrolyzable tannins) inhibit fecal pathogenic bacteria and to identify the potential bactericidal inhibitory activity on *Escherichia coli* O157:H7.

MATERIALS AND METHODS

Experimental design. An initial in vitro study (experiment 1) was conducted to assess the growth inhibition (experiment 1a) and bactericidal effects (experiment 1b) of the tannin preparations against *E. coli* O157:H7 in pure culture. Commercially available chestnut tannins (*Castanea sativa* Mill; containing about 80% hydrolyzable tannins) and mimosa tannins (*Acacia mearnsii,* black wattle; containing about 70% condensed tannins) (Chemtan, Exeter, N.H.) were tested *(18, 34, 38).* An in vivo study (experiment 2) was conducted to test the effect of chestnut tannin supplementation on fecal generic *E. coli* populations in the rumen cannulated steers fed Bermuda grass (*Cynodon dactylon*) hay. For the in vivo study, only the chestnut tannin was tested, because results from

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our initial experiment showed that it exerted a greater bactericidal effect on *E. coli* O157:H7 than the mimosa tannin.

Bacterial culture. *E. coli* O157:H7 strain 933 (ATCC 43895), strain 6058 (from Dr. Dan Rice, Field Disease Isolation Unit, Washington State University, Pullman), and strain 86-24 (from Dr. Francisco Diez-Gonzalez, Department of Food Science and Nutrition, University of Minnesota, St. Paul) were cultivated in an anoxic tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) medium at 37°C. Strain 933 was resistant to novobiocin and nalidixic acid (20 and 25 μg/ml, respectively), strain 6058 was resistant to rifampin (25 μg/ml), and strain 86-24 was resistant to streptomycin (100 μg/ml) *(3).* Each strain was maintained via aerobic growth in TSB medium (10 ml per tube) in screw-cap tubes (13 by 100 mm) at 37°C. Each strain had been grown (37°C) overnight in TSB medium and mixed (1 ml per strain) together before use in inoculations. A mixture of strains was chosen because *E. coli* O157:H7 strains have slightly different physiological characteristics and metabolism as well as ability to grow in the gut as a result of genetic divergence from the original O157. These three strains were chosen from a variety of human outbreaks linked to the consumption of ground beef and animal sources to represent the diversity within the strain of *E. coli* O157:H7. The mixture of these strains was used to verify that the treatment was not effective against a single strain of O157 and to elucidate any differences in the physiology of some representative strains of O157. They were mixed together to ensure that they all received identical treatments within the microenvironment. This allowed us to monitor specific strains of O157 following treatments to ensure that a treatment that kills one strain does not simply create a niche for another strain of O157 to utilize *(5).*

Experiment 1a: in vitro bacterial growth inhibition study. Overnight-grown *E. coli* O157:H7 strains 933, 6058, and 86-24 were mixed (1 ml per strain) and inoculated (0.2 ml; approximately 10^7 CFU) into crimp-top tubes (18 by 150 mm) containing 10 ml of TSB medium *(3)* supplemented with 0, 200, 400, 600, 800, and 1,200 μg of chestnut or mimosa tannin extract per ml. Tubes were cultured anaerobically under O_2-free N_2 gas at 37°C, and growth was determined via measurement of optical density at 600 nm at 30-min intervals in a Spectronic 20D+ model spectrophotometer (Spectronic Instruments, Rochester, N.Y.). The effect of tannins on the specific bacterial growth rate was determined by adding each tannin concentration to in vitro cultures of *E. coli* O157:H7 strains. Uninoculated tubes, receiving equivalent concentrations of tannins, served as blanks for the subtraction of background turbidity caused by tannin-protein interactions *(20).* Tannins were added to TSB as neutralized, filter-sterilized solutions prepared in water.

Experiment 1b: in vitro bactericidal analyses. In vitro bactericidal analyses were conducted with cultures treated with 0, 200, 400, 600, 800, and 1,200 μg of chestnut and mimosa tannins per ml at 6- and 24-h incubations in TSB medium. Samples taken from pure and mixed cultures were serially diluted (10-fold increments) in sterile phosphate-buffered saline (pH 7.0). Dilutions were plated (0.1 ml) on MacConkey agar (Difco, Becton Dickinson), MacConkey agar supplemented with novobiocin (20 μg/ml) and nalidixic acid (25 μg/ml), rifampin (25 μg/ml), and streptomycin (100 μg/ml) (for inoculated *E. coli* O157:H7 strains 933, 6058, and 86-24, respectively) and incubated overnight at 37°C *(3).* Colonies that grew on agar plates after 6- and 24-h incubations were directly counted (quantitative enumeration). Percent bactericidal effect was calculated from control to 1,200 μg of tannins per ml.

Experiment 2 (in vivo experiment). In an in vivo experiment, 12 rumen cannulated steers (Angus × Hereford × Brangus; *Bos* sp.; 544.2 ± 91 kg) were used to quantify generic fecal *E. coli* counting associated with different dose levels of tannins in steers fed Bermuda grass hay. Steers were housed in a common pen for 1 month (adaptation period) and randomly allocated to one of three treatments ($n = 4$ steers treatment^{-1}): (i) control (water infusion), (ii) 0.75% (7.5 g of tannins per day), and (iii) 1.5% (15 g of tannins per day; dry matter basis). The tannins were administered once daily (at about 0800 h) through the rumen cannula as premixes with warm water. Aseptic rectal fecal samples were collected (about 10 g per animal) after 1 month on a Bermuda grass hay diet, but prior to tannin addition, and served as a background sample on day 0 for each animal and on days 3, 6, 9, 12, and 15 for fecal *E. coli* analysis. Finally, all steers were offered Bermuda grass hay without tannin infusion for an additional 10 days after the cessation of tannin feeding, and they were sampled again (day 25) to reassess fecal generic *E. coli* concentrations. Generic *E. coli* concentrations were measured via the plating (100 μl per plate) of serial 10-fold dilutions of freshly collected feces (1 g of feces plus 9 ml of phosphate buffer) onto MacConkey agar plates after a 24-h incubation at 37°C. Generic fecal *E. coli* was identified by the method set forth in the *Laboratory Handbook on Bovine Mastitis (25),* and we counted colonies from pink to red, dry, and flat colonies (2 to 4 mm in diameter) surrounded by a pink zone of precipitated bile salts. Bile salt mixtures and crystal violet largely inhibit the growth of the gram-positive microbial flora *(37).* Cattle were weighed before the start of the experiments and after the experiments ended. The average daily weight gain was estimated by the final to initial weight difference.

Chemical analysis. The plant crude protein was determined by the Kjeldahl digestion procedure *(4).* Neutral detergent fiber, acid detergent fiber, and in vitro dry matter digestibility of forage samples were determined by the filter bag technique (ANKOM Technology Corp., Fairport, N.Y.).

Statistical analyses. Data from each experiment were analyzed by the MIXED procedure (SAS Institute, Cary, N.C.), and differences in mean log CFU per milliliter ($n = 2$) were determined by a repeated-measures analysis of variance, with the factors examined being dose levels and types of tannins, incubation time, and dose levels × types of tannins or incubation time interactions included in the model. The tannin dose level effects were tested by an orthogonal contrast for equally spaced treatments estimated by the MIXED procedure of SAS. The replicate (animal) was treated as a random variable. The *F*-test protected least-squares means procedure of SAS was used to separate treatment means. Percent growth inhibition rate and percent bactericidal effect were calculated by the highest dose level (1,200 μg/ml) of tannins to control (nontannins) difference at each incubation time. The specific growth rate was calculated as the maximum specific growth rate achieved by each culture *(20).*

RESULTS

Antibacterial activity of tannins (experiment 1a). The growth of *E. coli* O157:H7 strains in the absence or presence of different concentrations of tannins was tested in a medium. Bacterial growth was measured (Table 1), and the resulting growth curves were summarized by calculating the specific growth rates and plotting these values against the tannin concentrations tested (Fig. 1). The growth of *E. coli* O157:H7 strains was reduced ($P < 0.01$) in a

TABLE 1. *Growth rate of* Escherichia coli *O157:H7 in the presence of chestnut and mimosa tannins*[a]

Incubation time, h ($n = 2$)	Dose level (μg/ml): 0	200	400	600	800	1,200	% inhibition rate (0 vs 1,200 μg/ml)	*P* value
				Chestnut tannins				
0.0	0.0	0.0	0.0	0.0	0.0	0.0	—	0.0
0.5	0.04	0.04	0.01	0.06	0.0	0.05	—	0.50
1.0	0.04	0.07	0.01	0.07	0.05	0.05	—	0.49
1.5	0.06	0.09	0.02	0.09	0.05	0.01	—	0.38
2.0	0.19 A	0.19 A	0.12 B	0.15 A	0.18 A	0.19 A	—	0.07
2.5	0.36 A	0.33 AB	0.25 B	0.28 B	0.33 AB	0.29 B	19	0.05
3.0	0.52 A	0.45 AB	0.38 B	0.39 B	0.43 B	0.37 B	29	0.01
3.5	0.63 A	0.56 AB	0.50 B	0.54 B	0.57 AB	0.47 B	25	0.01
4.0	0.69 A	0.64 AB	0.59 B	0.61 B	0.60 B	0.52 B	25	0.05
SEM	0.024	0.09	0.01	0.165	0.06	0.01	—	—
				Mimosa tannins				
0.0	0.0	0.0	0.0	0.0	0.0	0.0	—	0.0
0.5	0.01	0.06	0.01	0.04	0.12	0.01	—	0.43
1.0	0.10	0.06	0.10	0.10	0.10	0.02	—	0.25
1.5	0.15 A	0.09 AB	0.04 B	0.12 A	0.12 A	0.08 AB	47	0.05
2.0	0.26 A	0.21 AB	0.10 B	0.12 B	0.12 B	0.12 B	54	0.05
2.5	0.40 A	0.34 AB	0.23 B	0.23 B	0.23 B	0.21 B	48	0.01
3.0	0.52 A	0.47 AB	0.36 BC	0.38 BC	0.34 BC	0.25 C	52	0.01
3.5	0.65 A	0.55 AB	0.43 B	0.44 B	0.44 B	0.31 C	52	0.01
4.0	0.70 A	0.63 AB	0.49 B	0.46 B	0.46 B	0.43 B	39	0.01
SEM	0.008	0.01	0.05	0.03	0.07	0.06	—	—

	Linear	Quadratic	
Analysis of variance			
Chestnut tannins	0.001	0.10	
Mimosa tannins	0.001	0.01	
Interaction			
Source of tannins (ST)			0.003
Dose levels (DL)			0.001
ST × DL			0.009
Incubation time			0.001
Incubation time × dose level			0.12
ST × DL × incubation time			0.74

[a] Growth rate (in hours) of *Escherichia coli* O157:H7 strains 933, 6058, and 86-24 during coculture in the presence of chestnut and mimosa tannins. Each strain had been grown separately overnight in tryptic soy broth medium at 37°C and mixed (1 ml per strain) together before inoculation into cultures ($n = 2$) supplemented with 0, 200, 400, 600, 800, and 1,200 μg of tannins per ml. Optical density at 600 mm was measured at 30-min intervals. Means within a row without a common letter differ ($P < 0.05$).

dose-dependent manner in the presence of chestnut and mimosa tannins during in vitro incubation (Table 1). Both linear and quadratic effects were significant ($P < 0.01$) for bacterial growth in the presence of mimosa tannins, while the bacterial growth inhibition rate exhibited a significant linear ($P < 0.001$) response with chestnut tannin infusion. When treatment effects were expressed as differences from the control treatment (0 versus 1,200 μg of tannins per ml), maximum growth inhibition rates of 29 and 54% were reached after 3 and 2 h of in vitro incubation in the presence of chestnut and mimosa tannins, respectively. Combining the data from both the chestnut and mimosa tannin treatments showed results that describe the concentration and incubation time-response relationships. There was a source of tannins × dose levels of tannins interactions ($P < 0.01$), and incubation time × dose level interactions tended to differ ($P = 0.12$), which indicated that antibacterial activity increased through time with mimosa tannins and that the maximum inhibition rate of growth occurred at 2 to 3 h and then remained relatively unchanged or decreased in inhibition rate, suggesting detoxification over time.

The addition of 200, 400, 600, 800, and 1,200 μg of tannins per ml significantly ($P < 0.01$) reduced the specific growth rate compared with the minus tannin control (Fig. 1). The specific growth rate was inhibited with increasing dose levels up to 800 μg of tannins per ml, but not at tannin concentrations of >1,200 μg/ml, and this inhibitory effect was relatively less ($P < 0.01$) in chestnut tannins.

Bactericidal effect of tannins (experiment 1b). The bactericidal effect of tannins varied among *E. coli* strains

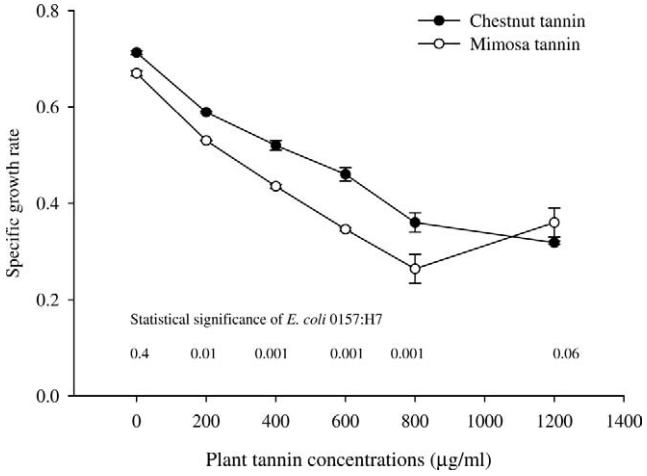

FIGURE 1. *Influence of tannin concentrations on the specific growth rate of* Escherichia coli *O157:H7 (*n *= 2) between chestnut and mimosa tannins.*

(Tables 2 and 3). The overall bactericidal effects of tannins on *E. coli* O157:H7 increased ($P < 0.01$) in a dose-dependent manner (linear effect; $P < 0.01$), but the response was quantitatively higher in chestnut tannins ($P < 0.01$) than in mimosa tannins. The addition of chestnut tannins after 6 h of in vitro incubation exhibited a linear bactericidal effect ($P < 0.06$ to $P < 0.001$) for most bacterial strains tested, whereas strain 86-24 had both linear ($P < 0.001$) and quadratic ($P < 0.02$) responses (Table 2). Neither strain 6058 nor 86-24 was affected by mimosa tannin infusion, but strain 933 was affected by mimosa tannin infusion in a linear ($P < 0.04$) and quadratic ($P < 0.01$) effect.

The bactericidal effect observed in cultures incubated for 24 h with the tannin preparations was similar to, although less than, that observed in samples collected from cultures incubated for 6 h (Table 3), thus suggesting the adaptation of the respective strains to the tannin exposure. The bactericidal effect of chestnut tannins on concentrations of *E. coli* O157:H7 increased ($P < 0.001$) in a dose-dependent manner. Although the bactericidal effect of chestnut tannins on strain 86-24 after 24 h of incubation was positively linear ($P < 0.001$) with mimosa tannin treatment ($P < 0.03$), the mimosa tannin treatment effect was a negative linear ($P < 0.03$; strain 933) response or relatively unchanged (strain 6058). There was an incubation time (6 versus 24 h of incubation; Tables 2 and 3) × dose level of tannin interaction ($P < 0.01$) and a source of tannins (chestnut versus mimosa tannins) × dose level interaction ($P < 0.03$) on bacterial concentrations, indicating that the bactericidal effect decreased during longer anaerobic incubation times; this bactericidal effect was relatively less in mimosa tannins. It must be noted that recovery on the plates supplemented with MacConkey agar as well as on the antibiotic-supplemented plates could result in the failure to recover some of the injured cells, meaning actual populations could be higher than those we detected.

In vivo experiment (experiment 2). Concentrations of fecal generic *E. coli* varied between sampling days (Fig. 2).

TABLE 2. *Bactericidal effect of tannin treatment* (n *= 2*) *on concentrations of* Escherichia coli *O157:H7 strains 933, 6058, and 86-24 after 6 h of treatment*[a]

E. coli O157:H7 strain	Dose level (μg/ml): 0	200	400	600	800	1,200	% BE (0 vs 1,200 μg/ml)[b]	SEM	*P* value: Linear	Quadratic
					Chestnut tannins					
933	5.1	4.8	4.9	4.6	4.4	4.3	16	0.03	0.001	0.32
6058	4.9	4.7	4.7	4.6	4.1	3.5	29	0.15	0.06	0.36
86-24	5.1	4.9	4.6	4.5	4.5	4.5	12	0.09	0.001	0.02
Mean	5.0	4.8	4.7	4.6	4.3	4.1	18	0.09	0.001	0.54
					Mimosa tannins					
933	5.0	4.7	4.5	4.4	4.4	4.6	8.0	0.09	0.04	0.01
6058	5.1	4.8	4.8	4.7	4.7	4.8	6.0	0.15	0.30	0.35
86-24	5.0	4.9	4.9	4.9	4.9	4.9	2.0	0.04	0.48	0.20
Mean	5.0	4.8	4.7	4.7	4.6	4.8	4.0	0.11	0.01	0.01
Overall mean	5.1	4.8	4.7	4.6	4.5	4.4	14	0.08	0.01	0.54
Interaction										
E. coli strain									0.05	
Source of tannins (ST)									0.01	
E. coli strain × ST									0.01	
Dose level (DL)									0.001	
E. coli strain × DL									0.63	
ST × DL									0.03	
E. coli strain × ST × DL									0.75	

[a] *E. coli* concentrations are in log CFU per milliliter. Each strain had been grown (37°C) overnight in tryptic soy broth (TSB) medium and mixed (1 ml per strain) together before inoculation. Cultures were treated with 0, 200, 400, 600, 800, and 1,200 μg of chestnut and mimosa tannins per ml at 6-h incubations in TSB medium and reinoculated (0.1 ml) in a MacConkey agar plate.

[b] Percent (%) bactericidal effect (BE) was calculated from control to 1,200 μg of tannins per ml.

TABLE 3. *Bactericidal effect of tannin treatment* (n = 2) *on concentrations of* Escherichia coli *O157:H7 strains 933, 6058, and 86-24 after 24 h of treatment*[a]

E. coli O157:H7 strain	Dose level (μg/ml): 0	200	400	600	800	1,200	% BE (0 vs 1,200 μg/ml)[b]	SEM	*P* value: Linear	Quadratic
Chestnut tannins										
933	4.9	4.8	4.8	4.7	4.6	4.7	4.1	0.9	0.01	0.48
6058	4.8	4.7	4.6	4.5	4.6	4.6	4.2	0.08	0.05	0.02
86-24	5.0	4.8	4.7	4.7	4.6	4.5	10.0	0.08	0.001	0.01
Mean	5.0	4.8	4.7	4.7	4.6	4.5	10.0	0.08	0.001	0.01
Mimosa tannins										
933	4.9	4.9	4.8	5.0	5.0	5.0	0.0	0.08	0.03	0.09
6058	4.9	4.9	5.0	4.9	4.9	5.1	0.0	0.10	0.38	0.76
86-24	4.8	4.7	4.8	4.8	4.6	4.5	6.2	0.08	0.03	0.08
Mean	5.0	4.8	4.7	4.7	4.6	4.8	4.0	0.10	0.89	0.29
Overall mean	5.0	4.8	4.7	4.7	4.6	4.5	10.0	0.06	0.01	0.09
Interaction										
E. coli strain									0.01	
Source of tannins (ST)									0.01	
E. coli strain × ST									0.01	
Dose level (DL)									0.001	
E. coli strain × DL									0.01	
ST × DL									0.03	
E. coli strain × ST × DL									0.15	

[a] *E. coli* concentrations are in log CFU per milliliter. Each strain was marked with a different antibiotic resistance trait to facilitate separate recovery. Strains had been grown (37°C) overnight in tryptic soy broth medium and mixed (1 ml per strain) together before inoculation into cultures supplemented with 0, 200, 400, 600, 800, and 1,200 μg of chestnut and mimosa tannin preparations per ml. Cultures were incubated for 24 h and then plated to MacConkey agar medium (nontannins).

[b] Percent (%) bactericidal effect (BE) was calculated from control to 1,200 μg of tannins per ml.

Concentrations of generic fecal *E. coli* patterns of nontannin-treated steers exhibited quadratic ($P < 0.05$) though multipeaked responses with sampling date. When chestnut tannin administration (15 g of chestnut tannins per day; dry matter basis) was infused intraruminally to steers, generic fecal *E. coli* shedding was lower on days 3 ($P < 0.03$), 12 ($P = 0.08$), and 15 ($P < 0.001$) when compared with animals that were fed similar diets without tannin supplementation (Fig. 2). Steers receiving 7.5 g of tannins per day (dry matter basis) exhibited intermediate decreases in fecal *E. coli.* After a 10-day secession of chestnut tannin administration, *E. coli* populations either stabilized at a new level or returned to the levels they had shown prior to tannin introduction.

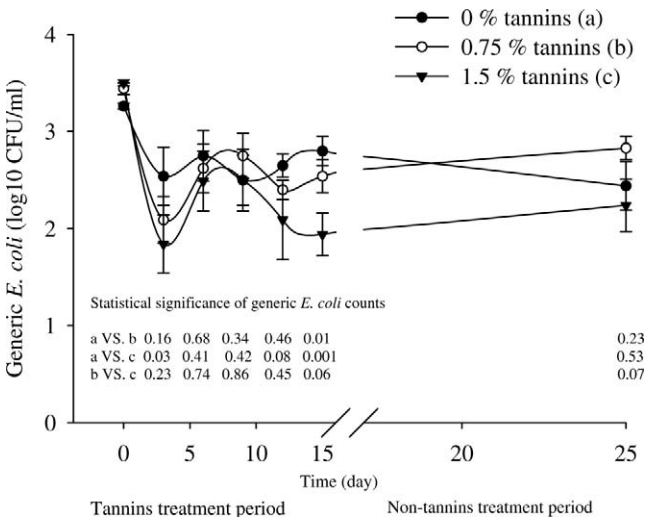

FIGURE 2. *Effects of chestnut tannin infusion on the generic fecal* E. coli *in steers (*n = *4) fed Bermuda grass hay. Steers were fed Bermuda grass hay during the first 15 days with chestnut tannin supplementation. Animals were again fed Bermuda grass hay after the cessation of chestnut tannin administration, and the last fecal samples were taken 10 days thereafter.*

Chemical compositions of the Bermuda grass hay diet for crude protein, neutral detergent fiber, acid detergent fiber, and in vitro dry matter digestibility were 11.4, 61.3, 31.7, and 66.6%, respectively (data not shown). There was no effect ($P = 0.18$) of tannin treatment on the steers' average daily weight gain (data not shown). The average daily weight gains in steers were 0.25, 0.14, and 0.31 kg per steer in the control, 7.5-g, and 15-g tannin per day treatment groups, respectively.

DISCUSSION

The principal objectives of this study were to measure and compare the antimicrobial activity of commercial plant tannins on *E. coli* O157:H7 and to determine if feeding tannins to steers had the potential to alter fecal generic *E. coli* concentrations. The main findings of the in vitro experiment are that chestnut and mimosa tannins had bacte-

riostatic and bactericidal effects in vitro against *E. coli* O157:H7; generally, mimosa tannins had greater bacteriostatic activity, and chestnut tannins had greater bactericidal activity.

Antibacterial and bactericidal effects of tannins in vitro. In the presence of tannins, the bacterial growth rate of *E. coli* O157:H7 was generally inhibited in a dose-dependent manner by both the chestnut and mimosa tannins, with these effects being more pronounced for mimosa tannins. However, the bactericidal effects of tannins on individual strains were reversed by the addition of chestnut tannins. The inhibitory effects of tannins from different tannin sources on various microorganisms have been demonstrated *(6, 22, 30).* Furthermore, tannins have been shown to alter rumen function and metabolism *(9, 29, 30).*

Condensed tannins from green tea have been shown to exert antimicrobial activity *(11, 15)* and to contain a mixture of catechin compounds. These tannin compounds inhibited the in vitro growth of *Staphylococcus aureus* ATCC 25932, *E. coli* K-12 strain G-6 *(15),* and other microorganisms *(7),* with *S. aureus* being more susceptible (183 μg/ml) than *E. coli* (1.1 mg/ml). It has been reported that methyl gallate and gallic acid (Galla Rhois tannins; Chinese sumac aphid, *Schlechtendalia chinensis* Bell), as sources of hydrolyzable tannins, have growth-inhibiting activities against both *E. coli* ATCC 11775 and *S. aureus* but not against *Bifidobacterium adolescentis* or *Lactobacillus acidophilus (1, 2).* Another study indicated that different levels of condensed tannins from sainfoin (*Onobrychis viciifolia* Scop) and bird's-foot trefoil (*Lotus corniculatus*) inhibited the growth and proteolysis of *S. bovis, B. fibrisolvens, Fibrobacter succinogenes, Ruminococcus albus,* and *R. amylophilus (16, 20).* These inhibitory mechanisms have been shown to disrupt membrane integrity *(14, 15)* or to cause iron depletion *(13, 17, 30).* Chung et al. *(6)* reported that the growth inhibition of *E. coli* by tannic acid (as a source of hydrolyzable tannins) was restored by the supplementation of additional iron, thus indicating that some tannins chelate iron or otherwise make it unavailable to the microorganisms. Plant tannins may also affect enzymes of intestinal bacteria *(6, 22, 30).*

In the present study, the maximum inhibition of the *E. coli* growth rate caused by tannins was reached 2 to 3 h after anaerobic incubation and then remained relatively unchanged or decreased, indicating detoxification or microbial adaptation over time. These findings agree with the data of Smith et al. *(32),* who reported that increasing the oxidative stress response allows *E. coli* strains to overcome the inhibitory effect of condensed tannins (wattle tannins) in an anaerobic but not an aerobic medium. Recently, Smith et al. *(32)* reported that the effect of condensed tannins (black wattle, *A. mearnsii*) on *E. coli* BW13711 appeared to be bacteriostatic rather than bactericidal at 0.1% wattle tannins, but at a higher tannin concentration of 0.2%, it appeared to be bactericidal. The site(s) and number of hydroxyl (–OH) groups on the tannins are possibly related to their relative toxicity to microorganisms, with evidence suggesting that increased hydroxylation results in increased toxicity *(10).* Chestnut (hydrolyzable) tannins consist mainly of vescalagin and castagin molecules, which have three –OH groups *(31),* whereas mimosa (condensed) tannins are composed of catechin and gallocatechin molecules, which have two –OH groups *(38),* indicating that increased hydroxylation results in increased microbial toxicity. However, both catechol (two –OH groups) and pyrogallol (three –OH groups) are hydroxylated phenols and have been shown to be toxic to microorganisms *(10).* Results from the present study show that the overall bactericidal effect of tannins on *E. coli* O157:H7 was increased in a dose-dependent manner, but quantitatively greater inhibition was observed with chestnut tannins than with mimosa tannins.

Under transmission and field emission scanning electron microscopy, *S. bovis* was seen to show increased chain formation and clumping when the tannic acid concentration exceeded 0.2% *(27).* When the tannic acid concentration was increased to 0.75%, cell lysis increased, and abnormal cells appeared. In contrast, *Streptococcus gallolyticus* (*Streptococcus caprinus*) cells were unaffected by the presence of tannic acid. These data and the present study indicate that different bacterial strains counteract the bactericidal or antibacterial activities of tannins isolated from different plants by a number of different mechanisms. One mode of action of plant tannins in antimicrobial activity is to complex with dietary nutrients through hydrogen and hydrophobic effects, as well as by covalent bond formation *(13, 30).* Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, and minerals *(30).* However, the antimicrobial significance of this particular activity has not been explored.

In vivo experiment. It would be interesting and potentially very beneficial to animal nutrition and health to determine the mechanisms by which tannins inhibit the growth of fecal *E. coli* and the bacterial-tannin interactions that take place to inhibit growth. The present in vivo study shows that steers receiving chestnut tannins had lower fecal generic *E. coli* populations than steers receiving no tannins in their diet. These results suggest that the addition of chestnut tannins is efficacious in decreasing the fecal *E. coli* in steers fed Bermuda grass hay. Chestnut tannins, primarily hydrolyzable tannins, can be degraded in the anaerobic environment of the intestinal tract *(26, 31).* However, the mechanisms of action of chestnut tannins on fecal generic *E. coli* in steers are not known, but previously described results from condensed tannins are possible evidence of their protective effect. Condensed tannins from green tea or legume forages were shown to affect gastrointestinal bacterial populations in humans *(28),* pigs *(11),* chickens *(12),* rats *(33),* and ruminants *(21).* In the human study, a period of 4 weeks of consuming condensed tannins from green tea was necessary to decrease counts of total *Clostridium* species, including *Clostridium perfringens (28).* In pigs, a diet containing 0.2% tea tannins for 2 weeks resulted in significantly increased levels of lactobacilli and a decrease in the levels of total bacteria and *Bacteroidaceae* in the feces *(11).* However, the role of competitive interactions

among fecal *E. coli* strains within the gastrointestinal tract is unknown at this time, specifically, whether tannin delivery is through the tannin-containing plant itself or through some feed supplement that would be chewed several times and then passed through the rumen before reaching the small and large bowel and recto-anal mucosal junction, where most *E. coli* O157:H7 is found to colonize and adhere *(8).*

Infusion of chestnut tannins through the rumen cannulae of steers had no effect on average daily weight gain. Supplementation of sheep and goat diets with 10% chestnut tannins reduced dry matter and fiber digestibility but did not affect the animals' body weight or body condition scores *(39).* However, chestnut tannins may form chelates with iron, which influences the reabsorption of the metal in the animal digestive tract *(18).* Min et al. *(23)* reported that the daily infusion of quebracho condensed tannin (2% tannins per kg of dry matter intake) through the rumen cannulae of steers grazing winter wheat (28 g of crude protein per 100 g of dry matter) forage improved animal performance. They went on to suggest that the combination of increasing bypass protein flow to the small intestine and decreasing frothy bloat and methane production led to the 15% increase in average daily gain observed with condensed tannin supplementation.

It was concluded that chestnut and mimosa tannins have growth-inhibitory and bactericidal effects in vitro against *E. coli* O157:H7; quantitatively, mimosa tannins had higher growth-inhibitory activity, but chestnut tannins had greater bactericidal activity. The mechanisms for increasing bactericidal activity in chestnut tannins on pathogenic bacterial strains are unknown, and the potential value of tannins for reducing pathogenic bacterial populations in steers needs to be further tested by an oral delivery system rather than by direct infusion into the rumen and increasing the time period over which tannins are fed. Experimental animals from each group should be housed separately in the future study to minimize possible cross-contamination.

ACKNOWLEDGMENTS

The authors thank the Beef Initiative Program of the Texas Agricultural Experiment Station for funding this research. Appreciation is expressed to laboratory technicians Ann Marie Prazak, Kate Andrews, and Jim Snodgrass of the U.S. Department of Agriculture, Agricultural Research Service, Food and Feed Safety Research Unit, College Station, Texas, for their assistance in laboratory analysis.

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