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Effect of Diet on the Shedding of *Escherichia coli* O157:H7 in a Sheep Model

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The purpose of this study was to develop a sheep model to investigate reproduction, transmission, and shedding of *Escherichia coli* O157:H7 in ruminants. In addition, we investigated the effect of diet change on these parameters. Six groups of twin lambs given oral inoculations of 10^5 or 10^9 CFU of *E. coli* O157:H7 and their nondosed mothers were monitored for colonization by culture of fecal samples. A modified selective-enrichment protocol that detected *E. coli* O157:H7 at levels as low as 0.06 CFU per g of ovine feces was developed. Horizontal transmission of infection occurred between the lambs and most of the nondosed mothers. When animals were kept in confinement and given alfalfa pellet feed, lambs receiving the higher dose shed the bacteria sooner and longer than all other animals. However, when the animals were released onto a sagebrush-bunchgrass range, every animal, regardless of its previous status (dosed at one of the inoculum levels tested or nondosed) shed *E. coli* O157:H7 uniformly. Shedding persisted for 15 days, after which all animals tested negative. *E. coli* O157:H7 reproduction and transmission and the combined effect of diet change and feed withholding were also investigated in a pilot study with experimentally inoculated rams. Withholding feed induced animals to shed the bacteria either by triggering growth of *E. coli* O157:H7 present in the intestines or by increasing susceptibility to infection. Introduction of a dietary change with brief starvation caused uniform shedding and clearance of *E. coli* O157:H7, and all animals then tested negative for the bacteria. This work suggests that appropriate preharvest management to control diet may significantly reduce the risk of *E. coli* O157:H7-positive animals going to slaughter.

Enterohemorrhagic strains of *Escherichia coli*, especially those of the serotype O157:H7, have been implicated in human hemorrhagic colitis, hemolytic-uremic syndrome, and thrombotic thrombocytopenic purpura (13). It has been estimated that between 10,000 and 20,000 human infections with *E. coli* O157:H7 occur annually in the United States (11). Approximately 2 to 7% of the illnesses progress to hemolytic-uremic syndrome, which is characterized by hemolytic anemia, thrombocytopenia, renal failure, and a death rate of 3 to 5% (5). The pathogenesis of these strains of *E. coli* is due to at least two virulence factors: production of Shiga-like toxin(s) (SLT) and the formation of attaching and effacing lesions in the intestinal mucosa (29).

Outbreaks of infections in humans have been traced to the consumption of either contaminated undercooked beef, apple cider, or unpasteurized milk (1, 13). Following a number of surveys to detect *E. coli* O157:H7 in foods and animals, it has been established that dairy and beef cattle are reservoirs of *E. coli* O157:H7 (3, 14, 15, 25, 32). Although this *E. coli* serotype has not been implicated in animal disease, other serotypes of SLT-producing-*E. coli* (SLTEC) cause postweaning diarrhea in calves (O111:NM and O26:H11) (18, 19, 32) and the edema disease of swine (O138:K81 and O139:K12) (17, 27).

Fewer studies investigating the roles of other domestic or wild animals as reservoirs or their susceptibilities to *E. coli* O157:H7 have been done. Sheep, the second most commonly reared species of ruminant food animal, reportedly harbor SLTEC at high rates, despite the fact that serotype O157:H7 has not been reported to be present in their feces. For example, 67% of the sheep tested by Beutin et al. had detectable levels of non-O157:H7 SLTEC in their fecal material (2). When other investigators looked for contamination in retail sheep products, *E. coli* O157:H7 and other SLTEC serotypes were detected. Using DNA probes for SLT genes, Samadpour and colleagues did not detect serotypes but found that 48% of the lamb meat samples were positive for SLTEC (26). In another investigation, *E. coli* O157:H7 was recovered from 4 of 205 lamb meat samples analyzed (9). These studies suggest that sheep may have a role similar to that of cattle as a functional reservoir for *E. coli* O157:H7.

Our goal was to investigate the colonization and transmission of *E. coli* O157:H7 in a sheep model, which is a potentially more economical ruminant model than the cattle model. This is the first reported study in which sheep have been given an oral inoculum of *E. coli* O157:H7. We included lambs, as well as adult sheep, in our study because the few studies of cattle experimentally inoculated with *E. coli* O157:H7 showed a higher level of colonization of calves than of adult animals (8, 34). The purpose of this study was fourfold: (i) to establish a sensitive assay to detect *E. coli* O157:H7 in ovine feces, (ii) to determine the oral dose of *E. coli* O157:H7 required to establish intestinal reproduction in sheep, (iii) to determine whether a shedding animal could transmit the bacteria to a nondosed pen mate, and (iv) to determine if changes in diet would affect the shedding of *E. coli* O157:H7 by sheep.

MATERIALS AND METHODS

Experimental animals. This study was facilitated by our ability to dose twin lambs from the same ewe. In addition, the animal age was uniform in that all six sets of twin lambs were 14 ± 0.5 days old at the start of the study. We were able to house all animals in confinement under controlled conditions and later mon-

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TABLE 1. Experimental animals used in this study

Type of sheep	Group	Breed (sex)	Dose[a]
Ewes	I	Whiteface, Polypay	0
	II	Whiteface, Polypay	0
	III	Whiteface, Targhee	0
	IV	Whiteface, Polypay	0
	V	Whiteface, Polypay	0
	VI	Whiteface, Polypay	0
Lambs[b]	I A	Smutface (wether)	10^5
	I B	Smutface (female)	10^9
	II A	Smutface (female)	10^5
	II B	Smutface (wether)	10^9
	III A	Smutface (wether)	10^5
	III B	Smutface (wether)	10^9
	IV A	Smutface (wether)	10^5
	IV B	Smutface (wether)	10^9
	V A	Smutface (female)	10^5
	V B	Smutface (wether)	10^9
	VI A	Smutface (female)	10^5
	VI B	Smutface (female)	10^9
Rams		Polypay	10^9
		Polypay	0

[a] CFU of *E. coli* O157:H7 administered.
[b] All lambs were sired by Suffolk bucks.

itor them closely while they grazed on a sagebrush-bunchgrass range. The sheep used in this study included whiteface ewes, their smutface twin lambs, and Polypay rams. All animals and their dose levels are listed in Table 1.

(i) Ewes and lambs. While in confinement, each whiteface ewe and her smutface twin lambs were in a cement floor pen (1 by 2.5 m) inside a temperature-controlled building. Sheep had free access to water. Twice daily, sheep were offered alfalfa pellets and allowed ad libitum consumption. In addition, each animal was fed daily 115 g of a supplement (Cargill Nutrena Feeds; Billings, Mont.) containing calcium carbonate, bentonite (a clay used as a binding agent), salt, potassium chloride, sodium bicarbonate, a rumen ionophor (Bovatec), monocalcium, 20% crude protein (major constituents, wheat mill feed, soybeans, fish meal, meat, and bone meal), and vitamins A, D, and E. Twice daily, the animal pens were rinsed with water and all manure and feed particles were flushed into a drainage line which emptied into a 500-gal (ca. 2,000-liter) manure-settling tank.

To test the impact of feed change on the shedding of *E. coli* O157:H7, the animals were taken out of confinement. On day 32 of the study, the ewes and lambs were placed in a 36-ha pasture of native sagebrush-bunchgrass (elevation, 1,700 m). The dominant shrubs were sagebrush (*Artemisia tripartita*), bitterbrush (*Purshia tridentata*), horsebrush (*Tetradymia canescens*), and rabbitbrush (*Chrysothamnus nauseosus* Nutt.). Arrowleaf balsamroot (*Balsamorhiza sagittata*) was the most common forb. Bluebunch wheatgrass (*Agropyron spicatum*) was the dominant grass.

(ii) Rams. Adult Polypay rams were used in a pilot study to begin verifying the effects of dietary change, inoculum dose, and horizontal transmission in the shedding of *E. coli* O157:H7 seen in the larger lamb study. The ram study groups consisted of an inoculated animal and a nondosed pen mate. When the rams were being fed alfalfa pellets, they were penned together under conditions similar to those for the ewes and lambs described above except for the size of their pen (3 by 3 m). Their average dry-matter intake of alfalfa pellets was 3.15 kg per head per day. When feed was changed, the rams grazed on kochia weeds and crested wheatgrass.

Bacteria. ATCC 43894, an *E. coli* isolate that expresses both SLT type I and SLT type II, was used as a representative organism of the O157:H7 serotype (American Type Culture Collection [ATCC], Rockville, Md.). This strain was isolated by the Centers for Disease Control in Atlanta, Ga., in a case of hemorrhagic colitis in a human (21, 31). *E. coli* HB101, which expresses neither the O157 nor the H7 antigen, was used as a negative-control strain.

Inoculum preparation and administration. *E. coli* 43894 was grown overnight in Luria-Bertani (LB) broth (20) at 37°C, with aeration. Cell densities were determined spectrophotometrically at A_{600}, and estimated cell numbers were determined by serial dilution and spread plate culture. All determinations were done in triplicate. Cells were harvested by centrifugation, resuspended in skim milk medium (Difco Laboratories, Detroit, Mich.), and distributed into aliquots that contained 10^9 or 10^5 CFU. Aliquots were lyophilized for preservation, and viable cell numbers were determined by plate count on LB agar to be the same before and after lyophilization. The lyophilized cells were aseptically transferred into gelatin capsules (Torpac, Ltd., New York, N.Y.) and administered to animals in a single oral bolus. An alternative method, used to inoculate several lambs, was to dissolve the gelatin capsule in water prior to administration of the bacteria. No differences between these two methods of oral administration were detected.

Fecal sample and tissue collection. Fecal samples were obtained aseptically from adult animals (10 g) and lambs (1 g) by rectal palpation. Samples were collected prior to bacterial inoculation (predose), 6 h after the administration of the bolus (postdose), and then daily or weekly as indicated in Results below. Tissue specimens were collected postmortem from one ram that received 10^9 CFU of *E. coli* O157:H7 122 days prior to sacrifice. Two 10-g tissue samples from each of the following sites were aseptically collected. One sample was collected from the proximal end and one was collected from the distal end of the rumen, reticulum, omasum, abomasum, duodenum, cecum, and large intestine. All fecal and tissue specimens were immediately put into ice-cold transport medium (see paragraph below), packed in ice, and mailed by overnight express from the USDA Sheep Experiment Station to the University of Idaho.

Media and culture conditions. (i) Transport. For most applications, fecal samples were enriched for *E. coli* O157:H7 by using sterile Trypticase soy broth (BBL/Becton Dickinson, Detroit, Mich.) supplemented with cefixime (50 μg/l; Lederle Laboratories, Pearl River, N.Y.) (generously provided by D. D. Hancock, Washington State University), potassium tellurite (2.5 mg/l; Sigma Chemical Co., St. Louis, Mo.), and vancomycin (40 mg/l; Sigma) (referred to hereafter as TSB-CTV) (6, 13a, 35). For transport of these samples, specimens were collected and placed immediately in ice-cold TSB-CTV and processed within 24 h of collection. Nonenriched samples were collected in ice-cold sterile buffered glycerol saline solution (BGSS) prepared according to the method of Teague and Clurman (28).

(ii) Culture. Samples transported in TSB-CTV were enriched for *E. coli* O157:H7 by increasing the total culture volume with fresh TSB-CTV (2 to 5% [wt/vol]) and incubating with aeration at 37°C for 18 h. Following this selective-enrichment step, serial dilutions of each culture were made in sterile saline (0.15 M NaCl) and spread plates were prepared with sorbitol-MacConkey agar (Difco Laboratories) supplemented with cefixime (50 μg/l), potassium tellurite (2.5 mg/l), and 4-methylumbelliferyl-β-D-glucuronide (MUG) (100 mg/l) (Biosynth Ag Biochemica and Synthetica, Skokie, Ill.). This medium is referred to hereafter as SMAC-CTM. Samples transported in BGSS were not enriched for *E. coli* O157:H7 but were cultured by streak plate onto SMAC supplemented with MUG (100 mg/l) (SMAC-MUG). Cultures on both media were incubated overnight at 37°C and observed for sorbitol fermentation (pink-red colonies) and MUG utilization (fluorescence at 363 nm). Colonies that did not ferment sorbitol and did not use MUG were confirmed to be serologically identical to the inoculum strain, ATCC 43894, by using commercially available antisera (Pro-lab Diagnostics, Richmond Hill, Ontario, Canada).

Culture technique comparison. We compared the abilities of the modified selective-enrichment and the nonenrichment methods to recover *E. coli* O157:H7 purposely inoculated into animal feces. Three samples of fresh ovine feces (23 g each) were collected and inoculated with 55 ± 5 CFU (high inoculum), 5 ± 2 CFU (medium inoculum), 1 ± 0.1 CFU (low inoculum), or 0 CFU (no inoculum) of *E. coli* O157:H7. After bacteria were added, the fecal material was mixed to homogeneity with a mortar and pestle. All experiments were done in triplicate and dilutions in each experiment were plated in triplicate.

(i) Selective-enrichment protocol. To determine the optimal dilution of seeded feces for enrichment, 1 or 10 g of weighed seeded feces or a swab saturated with approximately 0.1 g of seeded fecal material was transferred into 7, 50, or 200 ml of TSB-CTV as indicated in Table 2. The inoculated media were incubated overnight at 4°C, to mimic transport conditions, and then processed as were the ovine fecal samples described above.

(ii) Nonenrichment. Approximately 0.1 g of inoculated feces on a swab or 1 g of inoculated feces was added to 7 ml of BGSS and incubated overnight at 4°C to mimic transport conditions. Samples were cultured on SMAC-MUG plates, incubated at 37°C for 18 h, and observed for sorbitol-negative and MUG-negative colonies.

Biochemical tests. The standard indole, methyl red, Voges-Proskauer, and citrate (IMViC) assays were used to differentiate *E. coli* from other isolates (10).

Disinfection. At the end of the study, all animal pens were disinfected with 0.5% hypochlorite acid. The same solution of hypochlorite acid was added to the manure-settling tank, and contents were mixed by air pressure. Fecal slurry from the holding tank was spread on the ground in an area to which humans and livestock had no access and treated with lime. By the end of the study, the manure-settling tank, the ground onto which the fecal slurry had been spread, and the animal pens were confirmed to be negative for *E. coli* O157:H7 by the selective-enrichment culture technique.

RESULTS

The animals in this study are listed in Table 1. All animals were healthy and free from disease at the onset of and throughout the study, even when they were actively shedding

TABLE 2. Evaluation of selective-enrichment and nonenrichment protocols for recovery of *E. coli* O157:H7 from ovine feces

E. coli O157:H7 inoculum (CFU/g) and protocol	Result with amt of fecal sample/vol of media[a]:			
	0.1[b]/7	1/7	1/50	10/200
None				
Enrichment	−[c]	−	−	−
Nonenrichment	−	−	−	−
Low (0.06)				
Enrichment	−	−	8 ± 1.5[d]	6 ± 1.5
Nonenrichment	−	−	ND[e]	ND
Medium (0.25)				
Enrichment	−	−	6 ± 1.0	4 ± 1.0
Nonenrichment	−	−	ND	ND
High (2.4)				
Enrichment	−	−	15 ± 0.7	11 ± 1.0
Nonenrichment	−	−	ND	ND

[a] Weight (in grams) of fecal sample per volume (in milliliters) of TSB-CTV (enrichment) or BGSS (nonenrichment).
[b] Culture medium was inoculated with a swab containing 0.1 g of feces.
[c] −, no *E. coli* O157:H7 detected.
[d] *E. coli* O157:H7 CFU ± standard deviation.
[e] ND, not determined.

E. coli O157:H7. No signs of diarrhea or unusual weight changes were detected.

Comparison of the selective-enrichment and nonenrichment protocols by using seeded feces. Serotype O157:H7-negative *E. coli* (HB101) gave the characteristic sorbitol-positive and MUG-positive results on SMAC-CTM or SMAC-MUG plates. Sorbitol-negative, MUG-negative colonies, characteristic of the serotype O157:H7, were identified as *E. coli* by the standard IMViC assay and confirmed as such by serology.

Some studies investigating *E. coli* O157:H7 have used nonenriched cultures (2, 15). Our results suggest that these techniques are much less efficient at demonstrating the presence of this organism in feces. *E. coli* O157:H7 was not recovered from ovine feces that were inoculated with levels of the bacterium as high as 2.4 CFU/g of feces when samples were assayed without enrichment by direct streaking onto SMAC-MUG plates without enrichment (Table 2).

In contrast, *E. coli* O157:H7 was consistently recovered from experimentally inoculated ovine feces, even at the lowest inoculum level tested, 0.06 CFU/g of feces, by the selective-enrichment culture protocol (Table 2). Interestingly, the enrichment technique enabled detection of *E. coli* O157:H7 at low inoculum levels (0.06 CFU/g of feces) only when 1-g samples were incubated in 50 ml of TSB-CTV, or when 10-g samples were incubated in 50 ml (data not shown) or 200 ml of TSB-CTV (Table 2). *E. coli* O157:H7 was not detected when less fecal material (0.1 or 1 g) was cultured in 7 ml of TSB-CTV. The ratio of fecal material to culture medium volume contributed to the sensitivity of this technique, presumably by providing conditions conducive to the growth of *E. coli* O157:H7. The larger medium volumes may inhibit overgrowth of the cultures by bacteria other than *E. coli* O157:H7 that were present in the original fecal inoculum. After overnight incubation in TSB-CTV, samples that were analyzed by serial dilution and spread plate culture on SMAC-CTM yielded from 3×10^6 to 1.6×10^7 CFU of *E. coli* O157:H7 per ml of culture. However, when these positive cultures were analyzed by streak plate culture on SMAC-MUG, a technique that does not efficiently dilute out background bacterial flora, only the 10-g samples that had been incubated in 200 ml of TSB-CTV yielded detectable *E. coli* O157:H7 colonies.

Recovery of *E. coli* O157:H7 from lambs and ewes following experimental infection. All lamb and ewe fecal samples were negative for *E. coli* O157:H7, by the selective-enrichment technique, before the lambs received an oral bolus containing 10^5 CFU (A twins) or 10^9 CFU (B twins) of *E. coli* O157:H7 (Table 3).

(i) Animals in confinement fed alfalfa pellets. Six ewes, each with her identical twin (A and B) lambs, were held, under controlled conditions, in confinement on a diet of alfalfa pellet feed to allow us to monitor shedding, horizontal transmission of infection, and the effect of this diet, relatively low in fiber compared with that on the native sagebrush and bunchgrass range, on these parameters. All twin B animals received an oral bolus of 10^9 CFU of *E. coli* O157:H7, and each twin tested positive for *E. coli* O157:H7 on at least one sampling while the animals were in confinement on a diet of alfalfa pellets (Table 3). Twin B in group I shed *E. coli* O157:H7 within 24 h of receiving the inoculation and was positive for the bacteria on days 4, 5, 6, and 12. This lamb shed *E. coli* O157:H7 for at least 12 days. Similarly, twin B in group III first tested positive for *E. coli* O157:H7 on day 4 and continued to shed the bacteria for at least 9 days. In contrast, the B twins in groups II and IV tested positive for the organism only once (days 12 and 26, respectively) while in confinement on a diet of alfalfa pellets. Both B twins in groups V and VI were first positive for the bacteria on day 5, and the group VI twin shed *E. coli* O157:H7 for at least 8 days. However, after day 5, the B twin in group V was consistently negative until day 26, when the animal again tested positive. The average length of time between inoculation and detectable shedding of *E. coli* O157:H7 by the B twins was approximately 8.8 days, and the average duration for which the animals tested positive was estimated to be 6 days (Table 3).

In the same 26-day period, only four of the six A twins, which received a single oral bolus containing 10^5 CFU of *E. coli* O157:H7, shed detectable levels of *E. coli* O157:H7 in their feces (Table 3). The earliest fecal sample from an A twin that was positive for *E. coli* O157:H7 was collected on day 12. That animal (twin A in group II) was the only A twin that tested positive for *E. coli* O157:H7 on two consecutive samplings (days 12 and 18) before testing negative. Fecal samples from three A twins (groups IV, V, and VI) tested positive at only one sampling, on day 26. The average length of time between inoculation and detectable shedding of *E. coli* O157:H7 by the positive A twins was approximately 22.5 days, and the average duration for which the animals tested positive was approximately 2 days (Table 3). Fecal samples from two twin A animals (groups I and III) were negative for the entire time the animals were on this diet.

Five of the six nondosed ewes were positive for *E. coli* O157:H7 26 days after their twin lambs were dosed with *E. coli* O157:H7, suggesting that the twins passed their infection to their mothers. On day 26, the last fecal sampling before animals were released from confinement onto a native sagebrush-bunchgrass range, three of the twin A lambs (groups I, II, and III), three of the twin B lambs (groups I, II, and III), and one ewe (group IV) were negative for *E. coli* O157:H7 (Table 3).

(ii) Animals unconfined on a native sagebrush-bunchgrass range. Animals were released from confinement onto a native sagebrush-bunchgrass range (on day 32) to determine the effect of the change from a relatively low-fiber to a high-fiber diet on sheep fecal *E. coli* O157:H7. On day 39, 7 days after the lambs and ewes were transferred from confinement to pasture, every animal tested positive for *E. coli* O157:H7 (Table 3). All animals again tested positive for the bacteria 8 days later, on

TABLE 3. Dietary influences on colonization, transmission, and shedding of *E. coli* O157:H7 among ewes and lambs

Group and members	Result on indicated day of fecal sampling with diet:																
	Alfalfa pellets									Sagebrush-bunchgrass					Alfalfa pellets		
	P[a]	h[b]	1	4	5	6	12	18	26	39[c]	47	62	89	104[d]	112[e]	116	117[d]
I																	
Twin A[f]	–[g]	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–
Twin B[h]	–	–	+[i]	+	+	+	+	–	–	+	+	–	–	–	–	–	–
Ewe	–	ND[j]	ND	ND	ND	ND	ND	ND	+	+	+	–	–	–	–	–	–
II																	
Twin A	–	–	–	–	–	–	+	+	–	+	+	–	–	–	–	–	–
Twin B	–	–	–	–	–	–	+	–	–	+	+	–	–	–	–	–	–
Ewe	–	ND	ND	ND	ND	ND	ND	ND	+	+	+	–	–	–	–	–	–
III																	
Twin A	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–
Twin B	–	–	–	+	+	+	+	–	–	+	+	–	–	–	–	–	–
Ewe	–	ND	ND	ND	ND	ND	ND	ND	+	+	+	–	–	–	–	–	–
IV																	
Twin A	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
Twin B	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
Ewe	–	ND	ND	ND	ND	ND	ND	ND	–	+	+	–	–	–	–	–	–
V																	
Twin A	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
Twin B	–	–	–	–	+	–	–	–	+	+	+	–	–	–	–	–	–
Ewe	–	ND	ND	ND	ND	ND	ND	ND	+	+	+	–	–	–	–	–	–
VI																	
Twin A	–	–	–	–	–	–	–	–	+	+	+	–	–	X[k]	X	X	X
Twin B	–	–	–	–	+	+	+	–	+	+	+	–	–	–	–	–	–
Ewe	–	ND	ND	ND	ND	ND	ND	ND	+	+	+	–	–	–	–	–	–

[a] P, predose.
[b] h, 6 h postdose.
[c] On day 32, animals were removed from confinement and feed was changed from alfalfa pellets to sagebrush-bunchgrass.
[d] Feed was withheld for 24 h prior to fecal sampling.
[e] Feed was changed to alfalfa pellets 24 h prior to fecal sampling.
[f] Every twin A received an oral dose of 10^5 CFU of *E. coli* O157:H7.
[g] –, no *E. coli* O157:H7 detected after selective enrichment at a detection limit of 1 CFU/10 g of feces.
[h] Every twin B received an oral dose of 10^9 CFU of *E. coli* O157:H7.
[i] +, 10^5 to 10^9 CFU of *E. coli* O157:H7 per ml of culture volume recovered after selective enrichment.
[j] ND, not determined.
[k] X, coyote fatality.

day 47. The average length of time the animals shed *E. coli* O157:H7 after their diet was changed to native sagebrush-bunchgrass was approximately 15 days. Feces from all animals tested negative for *E. coli* O157:H7 on each subsequent sampling (days 62, 89, 104, 112, 116, and 117). Twin A in group VI was a coyote fatality and hence could not be tested from day 104 (Table 3). Animals did not shed the bacteria, although food and water was withheld prior to collection of fecal samples, on days 104 and 117, and their diet was switched back to alfalfa pellets prior to collection of samples on day 112 (Table 3). This is in contrast to the effect of withholding feed seen in experiments with inoculated rams which were fed another diet (see below).

The effect of inoculum dose on the shedding of *E. coli* O157:H7 from adult rams. We compared the amount of fecal *E. coli* O157:H7 shed by adult rams given a single oral bolus of 10^5 or 10^9 CFU of *E. coli* O157:H7 with that shed by uninoculated animals. Predose samples from all rams in this study tested negative for the organism (Table 4). The ram given a dose of 10^5 CFU of *E. coli* O157:H7 shed the organism within 6 h postinoculation and continued to shed it for 1 day. On day 2, this animal tested negative, and it remained so for the following 13 days. The ram's nondosed pen mate remained negative throughout the study. Fecal samples from the ram inoculated with 10^9 CFU of *E. coli* O157:H7 tested positive for *E. coli* O157:H7 from day 1 postinoculation and continued to be positive through day 15 of the study (Table 4). Fecal samples from this ram's nondosed pen mate tested positive only briefly, on days 7 and 8 postinoculation, and tested negative thereafter, through day 15.

Effect of withholding feed and water on shedding of *E. coli* O157:H7 from adult rams following experimental infection. The adult ram dosed with 10^9 CFU of *E. coli* O157:H7 and its nondosed pen mate described above were monitored for 117 days (Table 5) to assess the effects of two variables (feed change and brief fast) on fecal *E. coli* O157:H7. Throughout this investigation, the rams were either in confinement on a diet of alfalfa pellets or grazing on kochia weed.

(i) Diet of alfalfa pellets. During the first 42 days postinoculation, the rams' diet was alfalfa pellets, and the dosed ram tested consistently positive for *E. coli* O157:H7 from day 1 through day 42. In contrast, the nondosed pen mate tested positive for the bacteria only briefly, on days 7 and 8, and tested consistently negative until day 42. Prior to testing on day 42, feed and water were withheld from both animals for 24 h,

TABLE 4. Effect of dose on colonization and transmission of *E. coli* O157:H7 in rams

Experimental animal	Result on indicated day of fecal sampling with *E. coli* O157:H7 inoculum (CFU):																		
	10^{5}[a]									10^{9}[a]									
	P[b]	h[c]	1	2	4	7	8	9	15	P[b]	h[c]	1	2	4	7	8	9	15
Dosed ram	−[d]	+[e]	+	−	−	−	−	−	ND[f]	−	−	+	+	+	+	+	+	+
Nondosed pen mate	−	−	−	−	−	−	−	−	ND	−	−	−	−	−	+	+	−	−

[a] Every dosed animal received the indicated amount of *E. coli* O157:H7 in a single oral administration.
[b] P, predose.
[c] h, 6 h postdose.
[d] −, no *E. coli* O157:H7 detected after selective enrichment at a detection limit of 1 CFU/10 g of feces.
[e] +, 10^5 to 10^9 CFU of *E. coli* O157:H7 per ml of culture volume recovered after selective enrichment.
[f] ND, not determined.

and fecal *E. coli* O157:H7 was detected from both the dosed and nondosed rams after this stress (Table 5).

(ii) Diet of kochia weeds. On day 43, the rams' diet was changed from alfalfa pellets to kochia weeds, and feed and water were withheld for 48 h prior to sampling on day 50 (Table 5). Both animals tested positive for *E. coli* O157:H7 on day 50 but thereafter consistently tested negative for the bacteria, except on day 92, when only the dosed ram again tested positive. Under these experimental conditions, it was not possible to determine if the change in diet or withholding feed influenced the shedding of *E. coli* O157:H7 from these animals.

On day 123 the rams were sacrificed, and by gross necropsy both rams appeared healthy and free of disease. Selective-enrichment culture of 14 tissue samples from the intestinal tract of the dosed ram gave negative results for *E. coli* O157:H7.

Quantitation of the recovery of *E. coli* O157:H7 from ovine feces. The *E. coli* O157:H7 cells shed by positive animals were not detected without selective enrichment. Analysis of 1 g of feces from positive animals (rams on days 42 and 50) by streak plating without enrichment (0.1 g of feces per SMAC-CTM plate) did not reveal *E. coli* O157:H7, despite the high counts (10^9 CFU/ml of TBS-CTV) obtained with the same samples when enrichment was used. However, we observed a pattern in the number of *E. coli* O157:H7 cells recovered after overnight enrichment culture of fecal samples. On the first day of shedding (Tables 3 and 5), numbers of *E. coli* O157:H7 in enrichment cultures ranged from 1×10^7 to 2×10^8 CFU/ml of TSB-CTV. On subsequent days and directly following dietary changes that increased the incidence of positive animals (withholding feed or changing the diet to native sagebrush and bunchgrass range), numbers of *E. coli* O157:H7 in enrichment cultures increased to 1×10^9 to 2×10^9 CFU/ml of TSB-CTV (Table 3, days 39 and 42; Table 5, days 42 and 50). The number of *E. coli* O157:H7 cells in the enrichment culture declined to 10^5 CFU/ml of TSB-CTV prior to the animals becoming negative. Although we processed all samples under similar conditions, we cannot rule out the possibility that slight variations in culture conditions (incubation time, temperature, and aeration, etc.) may have influenced the number of bacteria in the overnight enrichment cultures. Nonetheless, we believe that the animals shed the organisms, although at significantly lower numbers, in a pattern similar to that seen with the enrichment cultures.

Survival of *E. coli* O157:H7 in fecal slurry collected from experimental animals. While animals were in confinement, their collective fecal material was held in a common tank. *E. coli* O157:H7 survived in this collective fecal slurry for at least 6 weeks beyond the last addition of fresh fecal material, longer than any single animal shed the bacteria. The slurry was monitored both by selective-enrichment culture and by directly streaking the slurry onto SMAC-MUG plates. In contrast to the finding with fresh feces from positive sheep, *E. coli* O157:H7 cells were present in the fecal slurry in large enough numbers that they were easily detected without enrichment. This observation indicated that *E. coli* O157:H7 had multiplied in the slurry environment. However, after the slurry was treated at the end of the study, all slurry and ground samples tested negative for *E. coli* O157:H7.

DISCUSSION

The most significant finding of this study was that sheep apparently negative for *E. coli* O157:H7 were induced to shed the organism following dietary changes. Our results indicated that dietary change, by itself or in combination with withhold-

TABLE 5. Effect of diet on recovery of *E. coli* O157:H7 from rams

Experimental animals	Result on indicated days of fecal sampling with diet:														
	Alfalfa pellets										Kochia weeds				
	P[a]	h[b]	1	7	8	9	15	21	29	42[c]	50[d]	65	92	101[c]	107
Dosed ram[e]	−[f]	−	+[g]	+	+	+	+	+	+	+	+	−	+	−	−
Nondosed pen mate	−	−	−	+	+	−	−	−	−	+	+	−	−	−	−

[a] P, predose.
[b] h, 6 h postdose.
[c] Feed was withheld for 24 h prior to fecal sampling.
[d] Feed was withheld for 48 h prior to fecal sampling.
[e] An oral dose of 10^9 CFU of *E. coli* O157:H7 was given.
[f] −, no *E. coli* O157:H7 detected after selective enrichment at a detection limit of 1 CFU/10 g of feces.
[g] +, 10^5 to 10^9 CFU of *E. coli* O157:H7 per ml of culture volume recovered after selective enrichment.

ing of feed for 24 h, can cause sheep to excrete *E. coli* O157:H7. To arrive at this conclusion, we used a highly sensitive modified selective-enrichment culture technique that allowed us to detect *E. coli* O157:H7 at levels as low as 0.06 CFU/g of ovine feces. In addition, this study indicated that an oral bolus of 10^9 CFU of *E. coli* O157:H7 resulted in colonization and shedding of the bacteria for up to 92 days in adult sheep and that a shedding animal passed *E. coli* O157:H7 to a nondosed pen mate sheep. Throughout this study, we monitored animals for the presence of *E. coli* O157:H7 by culturing fresh fecal material.

In order to choose a method to detect *E. coli* O157:H7 in ovine feces, we modified a selective-enrichment protocol (6, 35) and compared its ability to recover the bacteria from the feces of experimental animals and from fresh *E. coli* O157:H7-negative feces into which the bacteria was seeded with that of a nonenrichment protocol. On the basis of our results, the selective-enrichment protocol was clearly more sensitive, by about 2 orders of magnitude, for the recovery of *E. coli* O157:H7 not only from seeded samples but also from dosed sheep and their nondosed pen mates. In fact, when feces from animals that were determined to be positive for *E. coli* O157:H7 by the modified selective-enrichment protocol were tested without enrichment, the samples tested negative. In addition, we determined that the amount of fecal material analyzed had significant bearing on whether *E. coli* O157:H7 was recovered from a given sample. In seeded fecal samples containing low levels of *E. coli* O157:H7 (0.06 CFU/g), it was imperative for detection of the organism that 1 to 10 g of material was analyzed. When less material, a swab of feces, from a known-positive sample was analyzed by the selective-enrichment technique, the sample tested negative. We also showed that the ratio of fecal material to selective-enrichment broth volume contributed to the sensitivity of the technique. For example, *E. coli* O157:H7 was consistently recovered from a seeded fecal sample when 1 g of feces was enriched in 50 ml of medium but not when it was enriched in 7 ml of medium. The ratio of fecal material to medium volume was important in providing conditions conducive to *E. coli* O157:H7 growth and in inhibiting overgrowth of the culture by other members of the fecal flora. For these reasons, we believe that our ability to detect *E. coli* O157:H7 shed from experimental sheep may have often been due to the great sensitivity of the assay we developed. We were confident that as little as 1 CFU of *E. coli* O157:H7 in a 10-g fecal sample could be detected by the selective-enrichment protocol modification we describe here.

Not many other studies have analyzed fecal samples by this intensive procedure. In most surveys to detect shedding of *E. coli* O157:H7 in animals, a swab of fecal material has been cultured. For example, in a study of 1,266 dairy cattle, Wells et al. enriched feces collected on swabs in modified TSB, plated growth onto SMAC, and serotyped potential colonies using O and H antisera (32). They recovered *E. coli* O157:H7 from 2.3% of the calves, 3% of the heifers, and 0.15% of the adult cows. Similarly, Hancock and colleagues (14) have analyzed over 6,000 rectal swab samples from dairy and beef cattle by incubating them in TSB supplemented with vancomycin. The percentage of animals positive for *E. coli* O157:H7 by their culture methods was similar to the percentages found by Wells et al., except that the incidence among dairy calves was much higher, at 9.6%. The only study in which large amounts of fecal material were analyzed for *E. coli* was reported by Samadpour et al. (25). These investigators cultured 10-g fecal samples in 90 ml of modified TSB and determined the incidence of SLTEC in dairy calves. The only survey study that has included sheep was done by Beutin et al., who determined the prevalence of SLTEC in seven species of domestic animals (2). In that study, rectal swabs were inoculated onto LB agar, endo agar, and blood agar plates without enrichment. Colonies characteristic of *E. coli* were identified biochemically, serotyped, and determined to be SLT positive by colony hybridization. *E. coli* O157:H7 was not among the serogroups detected in sheep.

The study in which we monitored six groups of maturing twin lambs given oral inoculations of *E. coli* O157:H7 and their nondosed pen mate mothers has several implications. The inoculum levels of 10^5 and 10^9 CFU of *E. coli* O157:H7 were chosen on the basis of previous reports of experimental oral inoculation of calves with SLTEC (34). The inoculum dose given to the lambs influenced the duration of shedding and the level at which *E. coli* O157:H7 was shed in their feces. On average, lambs receiving a high dose of *E. coli* O157:H7 were positive for the organism almost 2 weeks sooner and for at least 4 days longer than lambs that received a lower inoculum while in confinement on a diet of alfalfa pellets. There may have been horizontal transmission between lambs, and we could not rule out the possibility that twins given a low dose may have acquired their *E. coli* O157:H7 from their dosed B twin pen mate. Horizontal transmission from the dosed lambs to their nondosed mothers was apparent, and by 26 days postinoculation, five of the six ewes were overtly shedding *E. coli* O157:H7.

We tested the effect of diet, as feed change and feed withdrawal, on shedding of this pathogen by the experimental group of sheep. The ewe and lamb diet was changed from alfalfa pellets to native sagebrush-bunchgrass. This diet change also entailed changes in location (confinement to pasture), potential levels of intake, and other variables associated with these differences. Within 7 days of this diet change, all animals in the study tested positive for *E. coli* O157:H7. Because the animals were no longer in close penned quarters, horizontal transmission of *E. coli* O157:H7 between animals was likely to be reduced. This suggests that although several animals tested negative for *E. coli* O157:H7 just before this dietary change, they may have actually been colonized with the bacteria. Presumably, the diet change induced selective microbial growth in the intestine so that the level of *E. coli* O157:H7 shed became detectable. Alternatively, the diet change may have increased animal susceptibility to colonization with *E. coli* O157:H7 so that horizontal transmission was increased even though the animals were no longer penned. Once on a diet of native sagebrush-bunchgrass, the animals continued to shed the bacteria for more than three times as long as the average length of time that positive animals shed the bacteria while being fed alfalfa pellets (15 versus 4 days). After this period of shedding, all animals reverted and tested negative for *E. coli* O157:H7. The animals remained negative although we introduced several dietary changes, which included withholding feed for 24 h and reconfining animals in drylots and feeding them an alfalfa pellet diet.

We hypothesize that diets high in nutrients and low in fiber induce a lower incidence of transmission and/or shedding of fewer *E. coli* O157:H7 cells but do not induce clearance of the organism from the intestine. Conversely, diets low in nutrients and high in fiber and briefly withholding feed both induce shedding of larger numbers of *E. coli* O157:H7 and/or increased susceptibility to new intestinal colonization but also induce elimination of the organism. The mechanism of these dietary effects may include changes in volatile fatty acid (VFA) concentrations. Diets with increased fiber content and withholding feed are both known to decrease the VFA concentrations in the ruminant gut and affect bacterial colonization patterns (4, 7, 12, 30). In vitro studies have shown that *E. coli*

O157:H7 grows well in bovine rumen fluid from animals which have fasted for 24 h, while its growth is restricted in bovine rumen fluid from a well-fed animal (24, 33). In addition, low VFA concentrations are seen during the progression from preruminant to ruminant, and the enterobacteria predominate until the normal microbiota is established (22).

We observed that animals appearing negative for *E. coli* O157:H7 while on the relatively low-fiber–high-nutrient alfalfa pellet diet became positive for the bacteria when their diet was changed to the relatively high-fiber-low-nutrient diet of sagebrush-bunchgrass. Although we did not measure fiber components of the two diets, previous studies in which a similar sagebrush-bunchgrass pasture and alfalfa pellets were analyzed found the neutral detergent fiber contents to be 58 and 47%, respectively (16, 23). We also did not measure VFA concentrations while the animals were on these diets. However, it can be concluded that immature forage, increasing levels of energy and protein, increased levels of intake, and pelleting roughage tend to increase ruminal VFA production and concentrations (7). Given the differences in neutral detergent fiber content between alfalfa pellets and native range and the potentially lower protein content, digestion coefficient, and feed intake for the native range compared with alfalfa pellets, we speculate that ruminal VFA concentrations were higher when the sheep were fed alfalfa pellets than when they were allowed to graze the native sagebrush-bunchgrass range.

During the course of this investigation, the lambs progressed from preruminant (2 weeks old at the onset of the study) to ruminant (17 weeks old at the end of the study). This progression may also have affected their shedding of *E. coli* O157:H7, and, in support of this notion, several lambs were positive for the bacteria for the first time as late as 26 days postinoculation. Thus, although our findings most likely reflect dietary induction of increased intestinal *E. coli* O157:H7 growth and/or increased susceptibility to horizontal transmission, we cannot rule out the possibility that natural maturation and acquisition of their ruminant microbial community may also have contributed to the shedding by lambs after their diets were changed to sagebrush-bunchgrass. Apparently, once the lambs and ewes shed the *E. coli* O157:H7 from their gut, they were free of the organism and were unaffected by conditions that may have altered their intake of fiber or ruminal VFA. Withholding feed or changing their diet to alfalfa pellets did not change their *E. coli* O157:H7-negative status.

We also report here a pilot study with adult rams to begin verifying the effects of inoculum dose, horizontal transmission, and dietary change in the shedding of *E. coli* O157:H7 seen in the larger lamb study. As observed with the lambs and ewes, the ram dosed with 10^9 CFU of *E. coli* O157:H7 showed prolonged shedding of *E. coli* O157:H7 (15 days) and transmitted its infection to its nondosed pen mate. In contrast, the ram dosed with fewer bacteria (10^5 CFU of *E. coli* O157:H7) shed only briefly and did not pass the bacteria to its nondosed pen mate.

The ram dosed with 10^9 CFU of *E. coli* O157:H7 and its nondosed pen mate were monitored for more than 3 months to analyze the combined effects of both feed withdrawal and feed change. While animals were on the alfalfa diet, feed was withheld for 24 h, following which both the positive dosed ram and the negative nondosed ram shed *E. coli* O157:H7. Feed was also withheld after the rams had been on a kochia weed diet for several days. As predicted by our hypothesis, the animals shed and cleared all the *E. coli* O157:H7 organisms from their gut. Fecal samples from both rams initially tested positive for *E. coli* O157:H7 after feed was withheld and then became negative. We established that the rams were negative for *E. coli* O157:H7 by fecal and intestinal tissue culture.

Thus, diet content, either by itself or in combination with feed withdrawal, appears to play a significant role in the clearance of *E. coli* O157:H7, which may transiently colonize the ruminant gut. This study suggests that feed withdrawal may induce apparently *E. coli* O157:H7-negative animals to become positive for the bacteria either by triggering growth of *E. coli* O157:H7 present in the gastrointestinal tract or by increasing susceptibility to colonization. In addition, although a high-fiber diet may predispose animals to initial colonization with *E. coli* O157:H7, it also induces the rapid elimination of the organism from the gut. Studies to expand our investigations of the effect of diet on shedding and transmission of *E. coli* O157:H7 in sheep and to determine if our finding will have similar implications for cattle are ongoing in our laboratory. Appropriate preharvest management to control diet and feed withdrawal may significantly reduce the risk of *E. coli* O157:H7-colonized livestock going to the slaughterhouse.

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