

Displacement of *Escherichia coli* O157:H7 from Rumen Medium Containing Prebiotic Sugars†

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A fed-batch, anaerobic culture system was developed to assess the behavior of *Escherichia coli* O157:H7 in a rumen-like environment. Fermentation medium consisted of either 50% (vol/vol) raw or sterile rumen fluid and 50% phosphate buffer. Additional rumen fluid was added twice per day, and samples were removed three times per day to simulate the exiting of digesta and microbes from the rumen environment under typical feeding regimens. With both types of medium, anaerobic and enteric bacteria reached 10^{10} and 10^4 cells/ml, respectively, and were maintained at these levels for at least 5 days. When a rifampin-resistant strain of *E. coli* O157:H7 was inoculated into medium containing raw rumen fluid, growth did not occur. In contrast, when this strain was added to sterile rumen fluid medium, cell densities increased from 10^6 to 10^9 CFU/ml within 24 h. Most strains of *E. coli* O157:H7 are unable to ferment sorbitol; therefore, we assessed whether the addition of sorbitol as the only added carbohydrate could be used to competitively exclude *E. coli* O157:H7 from the culture system. When inoculated into raw rumen broth containing 3 g of sorbitol per liter, *E. coli* O157:H7 was displaced within 72 h. The addition of other competitive sugars, such as L-arabinose, trehalose, and rhamnose, to rumen medium gave similar results. However, whenever *E. coli* O157:H7 was grown in sterile rumen broth containing sorbitol, sorbitol-positive mutants appeared. These results suggest that a robust population of commensal ruminal microflora is required to invoke competitive exclusion of *E. coli* O157:H7 by the addition of “nonfermentable” sugars and that this approach may be effective as a preharvest strategy for reducing carriage of *E. coli* O157:H7 in the rumen.

The emergence of *Escherichia coli* O157:H7 as a human pathogen has led to renewed interest in the ecology of this organism in the bovine rumen and gastrointestinal tract (19). The presence of *E. coli* O157:H7 and other Shiga toxin-producing bacterial strains in the bovine rumen has not been well studied, but recent studies indicate low prevalence rates (22) of these organisms as well as low cell densities in the rumen (28). The low pH and high concentration of volatile fatty acids (VFA) ordinarily seen with the grain-rich rations used by the cattle industry act to limit growth of enteric bacteria in the bovine rumen (3, 25, 27), and as a result, these bacteria are considered to represent a minor and rather transient population (11, 14, 18).

However, despite the adverse growth conditions, the rumen is still widely considered to be an important reservoir of *E. coli*, and *E. coli* O157:H7 in particular, providing a continuous source of these bacteria into the intestinal tract of cattle (2, 11, 17). For these reasons, many of the strategies for decreasing the incidence of food-borne disease caused by *E. coli* O157:H7 are now focused on reducing carriage and shedding of the bacterium by cattle. Recently, changing the animals' diet from rolled corn (high starch) to alfalfa hay (low starch) was shown to decrease the number of acid-resistant *E. coli* in the fecal material (5, 20). Although it is not clear that *E. coli* O157:H7

is any more acid tolerant than commensal *E. coli* (1), the authors of this feeding study proposed that the dietary change would be effective in reducing fecal shedding of *E. coli* O157:H7. These findings were initially put in question by conflicting results (13); however, the results of Diez-Gonzalez et al. have since been substantiated by larger field trials (J. E. Keen, G. A. Ulrich, and R. O. Elder, 80th Conference of Research Workers in Animal Diseases, 1999, abstr. 86).

Strategies that make the ruminal environment more hostile for pathogenic *E. coli* to persist are also likely to offer preharvest management practices that could reduce the incidence of pathogenic *E. coli* in the food chain. Competitive exclusion strategies based on the administration of probiotic bacteria have been proposed as one way to reduce carriage of *E. coli* O157:H7 in cattle (8, 28). In principle, probiotic bacteria are introduced into the feed or provided as boluses, and either produce antimicrobial substances or otherwise outcompete *E. coli* O157:H7 in the rumen or colon.

Another option that could be effective would be to identify and provide prebiotics—carbohydrate substrates that selectively stimulate one or a limited number of commensal bacteria (4, 9). Prebiotic substrates would enrich for those organisms capable of metabolizing those substrates and displace those organisms incapable of prebiotic metabolism (9). It has recently been suggested that the inability of *E. coli* to reach high numbers in the rumen may, in fact, be due more to the success of commensal bacteria in competing for nutrients (7) than to inhibitory VFA concentrations.

The objectives of this study, therefore, were to compare growth of *E. coli* O157:H7 in a rumen-based medium containing selective substrates and to provide a theoretical basis for

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displacing *E. coli* O157:H7 from the bovine rumen by the use of prebiotic-like sugars.

MATERIALS AND METHODS

Bacterial strains. The commensal rumen strains of *E. coli* were isolated from freshly collected rumen fluid obtained from a single fistulated steer fed an alfalfa hay diet. Serially diluted rumen fluid samples were dispensed directly onto MacConkey-sorbitol (SMAC) agar, and colonies having typical *E. coli* morphologies were selected and transferred onto EMB agar. Presumptive *E. coli* colonies were transferred to Luria broth and Gram stained, and biochemical profiles were determined using API 20E identification strips (Bio-Mérieux, St. Louis, Mo.). Two strains having biochemical patterns consistent with *E. coli* were named *E. coli* AV8 and AV9. All *E. coli* O157:H7 strains were originally isolated from cattle and were obtained from C. Kaspar (University of Wisconsin). A rifampin-resistant (Rif^r) mutant of *E. coli* O157:H7 strain 965 was obtained by growing the organism in Luria broth containing up to 100 µg of rifampin per ml. Preliminary experiments showed that the general rumen bacterial population did not grow in media containing this antibiotic.

Growth in minimal medium. Since most strains of *E. coli* O157:H7 do not grow on sorbitol, the ability of a sorbitol-fermenting commensal strain to outcompete the former strain in minimal medium was assessed. Commensal strains AV8 and AV9 and *E. coli* O157:H7 965 were coinoculated into M9 minimal medium (22) at 10⁴/ml in the presence of either 0.2% glucose or 0.2% sorbitol, and growth and survival of the two strains under anaerobic conditions for 96 h was determined by plate counting (see below).

Fed-batch fermentations. Fed-batch fermentations in two types of rumen-based medium were performed. One medium consisted of 100 ml of autoclaved 0.2 M sodium phosphate buffer (pH 7.0) with 2.0 g of maltose and 1.0 g of starch per liter and 100 ml of raw (not sterilized) rumen fluid. The latter was obtained from rumen-cannulated animals (fed alfalfa or grass hay) 1 h after feeding. Large feed particles were first removed by passage through four layers of cheesecloth. The flasks (500-ml Erlenmeyer) were fitted with foam caps to allow gas exchange and placed in an anaerobic incubator (Forma Scientific, Marietta, Ohio) containing an atmosphere of 85% N₂, 10% H₂, and 5% CO₂ for 3 days. The medium was then inoculated with about 10⁵ CFU of *E. coli* O157:H7 965 (Rif^r) per ml and incubated at 38°C. Samples (10 or 30 ml) were removed three times per day, and 30 ml of sterile phosphate buffer-clarified rumen fluid (1:1) containing 2.0 g of maltose and 1.0 g of starch per liter were added twice per day (8:30 a.m. and 4:30 p.m.). In some experiments, the maltose-starch mixture was replaced by 3.0 g of sorbitol per liter.

The second medium consisted of equal volumes of sterile sodium phosphate buffer (as above) and sterilized rumen fluid, also containing 2.0 g of maltose and 1.0 g of starch per liter. The rumen fluid was sterilized by autoclaving at 121°C for 15 min. Flasks were inoculated with raw rumen fluid (5%, vol/vol; obtained from the same cow) and 10⁵ CFU of either *E. coli* O157:H7 965 (Rif^r) or the commensal strain, *E. coli* AV8, per ml. Samples were removed and sterile medium was added as described above. Each experiment was repeated at least two times.

Bacterial enumeration. The commensal and O157:H7 *E. coli* strains were enumerated on SMAC agar or SMAC agar containing 100 µg of rifampin and 2.5 µg of tellurite (SMACrt) per ml, respectively. Enteric bacteria were enumerated on EMB agar. The SMAC and EMB plates were incubated aerobically at 37°C for 48 h. Total anaerobes were enumerated on a medium consisting of tryptone (5.0 g/liter), yeast extract (2.5 g/liter), KCl (0.6 g/liter), NaCl (0.6 g/liter), MgSO₄ (0.5 g/liter), CaCl₂ (0.1 g/liter), trace element solution (10 ml), VFA mixture (10 ml/liter), vitamin solution (10 ml/liter), glucose (1.0 g/liter), cellobiose (0.25 g/liter), xylose (0.5 g/liter), ribose (0.5 g/liter), maltose (1.0 g/liter), agar (15 g/liter), and cysteine HCl (1.0 g/liter). The compositions of the trace element, VFA, and vitamin solutions have been described previously (15). The agar plates were poured in aerobic conditions and then held for several days (at least 4 days) in an anaerobic chamber (see above) before inoculation. Anaerobe samples were incubated at 38°C for 48 h. All dilutions were plated in duplicate.

RESULTS

Growth of commensal *E. coli* and *E. coli* O157:H7 in sorbitol minimal medium. Growth of a sorbitol-fermenting commensal *E. coli* strain and O157:H7 together in M9 minimal medium was measured during a 96-h incubation period. When glucose was the sole carbohydrate source, the strains showed similar

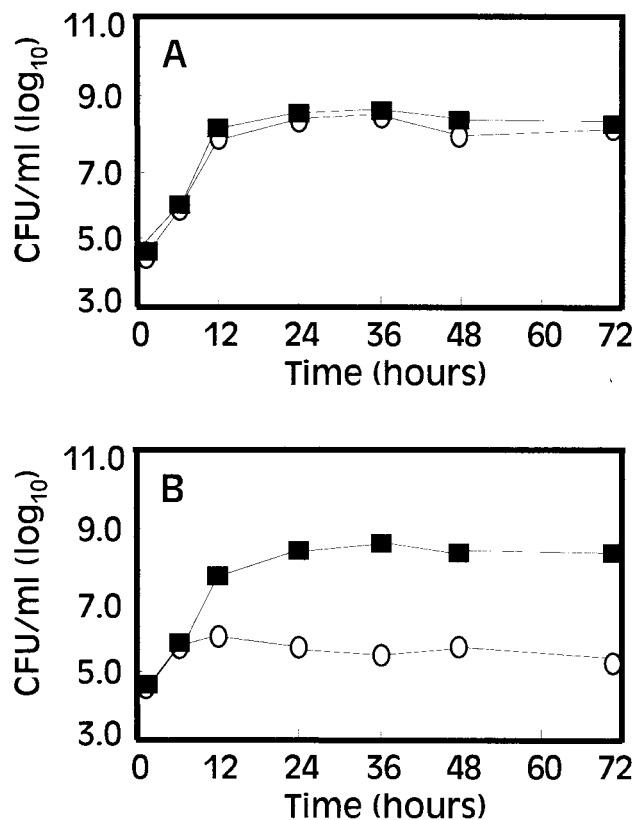


FIG. 1. Growth of commensal *E. coli* AV8 (■) and *E. coli* O157:H7 (○) in M9 medium containing 3 g of glucose (A) or sorbitol (B) per liter.

growth patterns and reached a final cell density of about 10⁸ CFU/ml after 12 h (Fig. 1A). However, when sorbitol (2 g/liter) was provided as the carbohydrate source, the commensal strain grew in a manner similar to that seen when glucose was added to the growth medium, but the O157:H7 strain did not grow, and its numbers were 3 orders of magnitude lower than that seen for glucose-containing medium (Fig. 1B).

Two other commensal and O157 strains gave the same results during growth in M9 medium. Similar effects were observed when sterile rumen fluid medium was used (data not shown), but in neither experiment was the O157:H7 strain eliminated from the fermentation medium. We did observe, however, that when the O157 strain was grown alone in sterile rumen fluid containing sorbitol, sorbitol-positive *E. coli* O157:H7 colonies eventually appeared (>96 h) on the SMACrt plates (data not shown). Sorbitol-fermenting derivatives did not appear in the M9 minimal medium.

Growth of enteric and anaerobic bacteria in rumen medium. When the culture medium contained a fresh rumen inoculum and was incubated under conditions representative of a high-grain diet, i.e., in the presence of 2.0 g of maltose and 1 g of starch per liter, there was little impact on the counts of enteric bacteria during the 72 h that measurements were made (Fig. 2). When the initial pH of the medium was set at pH 5.5 (by addition of HCl), the population of total anaerobes increased slightly, but by 72 h all three cultures had stabilized at about 10⁹ CFU/ml. In all subsequent experiments, the initial pH of

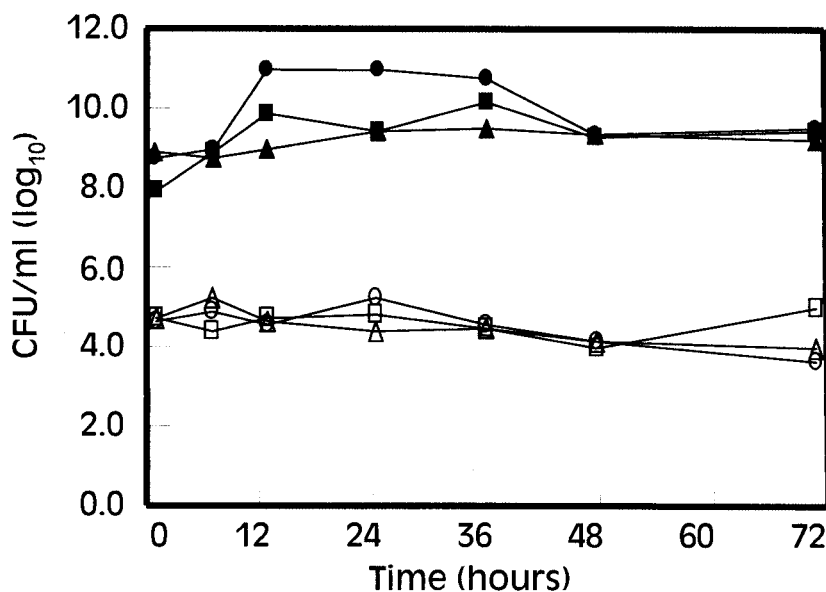


FIG. 2. Growth of total anaerobes (solid symbols) and enteric bacteria (open symbols) in raw rumen medium at pH 5.5 (●, ○), 6.0 (■, □), and 6.5 (▲, △).

the medium was adjusted to 6.5. During these experiments with raw rumen fluid, pH decreases of about 0.5 pH unit were observed, with most of the decrease occurring within the first 24 h of incubation (data not shown).

Growth of *E. coli* O157:H7 in the presence of rumen flora.

The number of enteric bacteria did not increase in the presence of the normal rumen microflora maintained in the fermentors, even at the higher pHs, suggesting that an actively growing population of ruminal microorganisms helps to restrict the growth of the enteric population. The effect of this background flora on growth of *E. coli* O157:H7, specifically, was then determined by growing *E. coli* O157:H7 strain 965 either in the raw rumen medium or in the same medium sterilized by autoclaving (Fig. 3).

In the presence of an active rumen flora, the number of O157:H7 either remained stable for the 72-h fermentation (at pH 6.0 and 6.5) or decreased below the limits of detection by 48 h (at an initial pH of 5.5) when the pH of the culture medium dropped below 5.0. However, in the absence of the rumen flora, the O157:H7 strain was able to grow to much higher total numbers, although the magnitude of the increase was also affected by the initial pH of the medium. At initial pHs of 6.0 and 6.5, *E. coli* O157:H7 levels exceeded 10^9 CFU/ml, compared to less than 10^7 CFU/ml at an initial pH of 5.5. Similar results were observed for *E. coli* O157:H7 strain 1275 (data not shown).

Use of nonfermentable sugars to competitively exclude *E. coli* O157:H7. Since *E. coli* O157:H7 was unable to grow in sorbitol-M9 medium (Fig. 1), we hypothesized that sorbitol could be used as a prebiotic, a substrate that would support growth of ruminal bacteria and competitively exclude *E. coli* O157:H7 from the simulated rumen fermentation flasks. In these experiments, 0.3% sorbitol was added to sterile rumen medium in lieu of glucose and starch. Treatments consisted of (i) raw rumen fluid as the inoculum; (ii) commensal strain AV8

with rumen flora; and (iii) Rif^r *E. coli* O157:H7 strain 965 with rumen flora.

With the rumen inoculum alone, there were no observed changes in the populations of anaerobic or enteric bacteria (Fig. 4A). Similarly, the commensal *E. coli* strain was also maintained at the inoculum level of 10^5 CFU/ml (Fig. 4B). In contrast, *E. coli* O157:H7 was displaced from the medium in the presence of the competitive rumen flora and sorbitol (Fig. 4C). In two separate trials, displacement occurred after 36 or 48 h, and in neither trial was *E. coli* O157:H7 detected after 72 h ($<10^2$ /ml). Interestingly, the displacement of the O157:H7 strain coincided with a commensurate increase in the number of total anaerobes in the fermentor. However, there was no measurable change in the population of total enteric bacteria.

To identify carbohydrates other than sorbitol that could be used as competitive exclusion sugars, fermentation profiles using API strips were performed on the commensal *E. coli* strains AV8 and AV9 and four O157:H7 strains (944, 965, 1275, and 1540). The results revealed several other differences in the fermentation patterns between commensal strains of *E. coli* and *E. coli* O157:H7. In particular, L-arabinose, trehalose, and rhamnose were utilized by the commensal *E. coli*, but slowly or not at all by the four *E. coli* O157:H7 strains. These sugars, along with sorbitol and esculin, a *p*-coumarin derivative previously shown to reduce carriage of *E. coli* O157:H7 (6), were each added (0.6 g/liter each, or 3.0 g/liter total) to sterile rumen medium, which was then inoculated with *E. coli* O157:H7 strain 965 (Rif^r) and/or 5% raw rumen inoculum.

In the absence of the rumen flora, *E. coli* O157:H7 grew to high cell density, but in the presence of the 5% rumen inoculum, *E. coli* O157:H7 was eventually eliminated from the culture (Fig. 5). There was no effect on the sorbitol-positive enterics or on the anaerobic population in either treatment (both were maintained at about 10^5 and 10^{10} CFU/ml, respectively). Although the rumen flora clearly acted as competition, even-

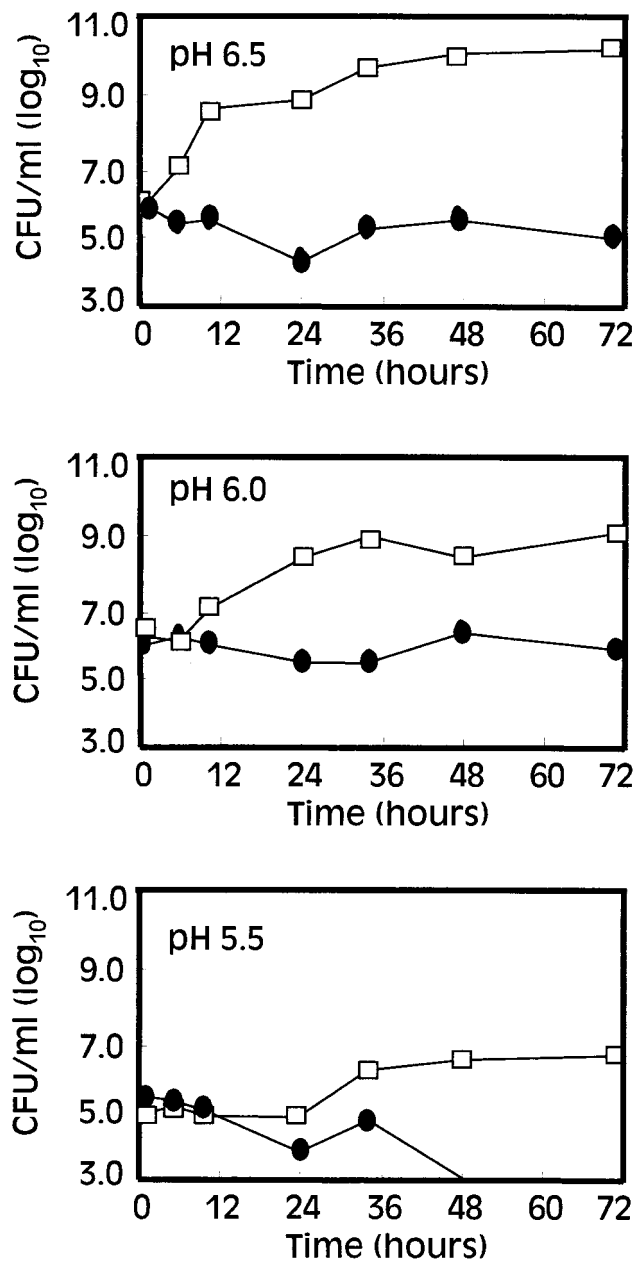


FIG. 3. Growth of *E. coli* O157:H7 in the presence (●) or absence (□) of rumen flora at pH 6.5, 6.0, and 5.5.

tually displacing the O157:H7 strain, the presence of the additional sugars did not appear to increase the rate at which displacement occurred compared to when sorbitol alone was present. When the *E. coli* O157:H7 strain was grown alone in the presence of the selective sugars, some sorbitol-positive mutants were also detected, but they emerged after 120 h of culture, compared to 72 h in the presence of sorbitol alone.

DISCUSSION

Although considerable effort has been devoted to manipulating the microflora of the rumen, the goal of most of these efforts has been to improve animal production, animal health,

and feed efficiency (19, 23, 26). Manipulation of the rumen flora to restrict growth of human pathogens has not been actively considered. The observed poor growth of commensal *E. coli* in rumen medium has led some investigators to consider ways to enrich for this organism and to identify factors responsible for poor rumen growth (12). Several environmental factors, including VFA, pH, temperature, oxidation-reduction potential, and the presence of bacteriophages, were individually evaluated for their ability to inhibit *E. coli* in a bovine rumen

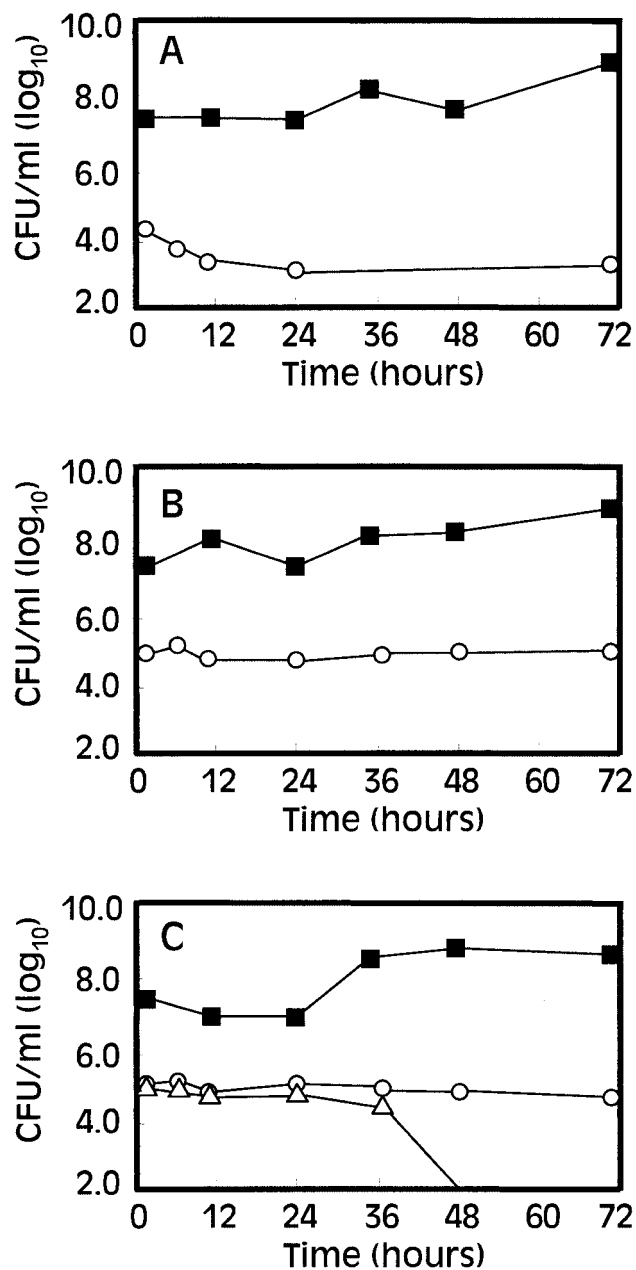


FIG. 4. Growth of total anaerobes (■), sorbitol-fermenting enteric bacteria (○), and *E. coli* O157:H7 (△) in sterile rumen medium containing sorbitol (3 g/liter) and a 5% rumen inoculum (A), 5% rumen inoculum and 10⁵ CFU of commensal *E. coli* AV8 per ml (B), and rumen inoculum and 10⁵ CFU of *E. coli* O157:H7 strain 965 per ml (C).

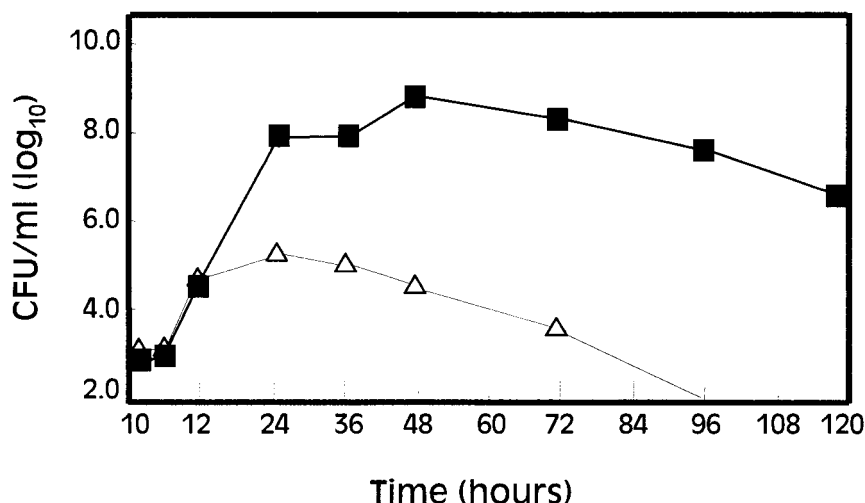


FIG. 5. Growth of *E. coli* O157:H7 in sterile rumen medium (■) containing sorbitol, L-arabinose, trehalose, rhamnose, and esculin (0.6 g/liter each) or in the same medium also containing a 5% raw rumen inoculum (△).

medium. However, none of these factors appeared to select against the establishment of *E. coli*.

In another early report, the ability of commensal strains of *E. coli* to ferment sorbitol led Wallace and coworkers (24, 25) to add this sugar to sheep rumen fluid in an effort to establish *E. coli* in this medium. Although enrichment of *E. coli* did not occur, presumably because of the high VFA concentration, these investigators argued that, with the right substrate, this strategy could still be an effective way to manipulate the rumen flora.

In the present study, we observed that growth of *E. coli* O157:H7 in a medium containing raw rumen fluid was also suppressed due to the presence of a competitive microflora. We speculated that even greater growth inhibition could be achieved by supplementing the medium with nutrients that only the indigenous flora, ordinarily present in rumen fluid, could utilize. This approach, to deprive *E. coli* O157:H7 of nutrients or to feed diets containing nutrients for which *E. coli* O157:H7 does not compete well, has recently been advocated as one of the most attractive means of restricting growth of this organism in cattle (7).

Since it has long been known that, in contrast to wild-type or commensal strains of *E. coli*, most strains of *E. coli* O157:H7 do not ferment sorbitol or do so slowly (16), sorbitol was used as a prebiotic, providing a competitive advantage for sorbitol-fermenting organisms, including the background rumen flora and commensal strains of *E. coli*. Although sorbitol added to raw rumen fluid did, as predicted, displace *E. coli* O157:H7 from the medium, we also observed that when *E. coli* O157:H7 was incubated alone in the presence of sorbitol in sterile medium, some sorbitol-positive mutants appeared. In none of our experiments with the competitive flora present, however, did sorbitol-positive *E. coli* O157:H7 cells appear before displacement had occurred.

To determine if other nonfermentable sugars could enhance the results observed by sorbitol, we identified several other sugars that were not well utilized by *E. coli* O157:H7 and included these sugars in the rumen medium. In the presence of the 5% raw rumen inoculum, a mixture of these additional

carbon sources plus sorbitol again led to exclusion of *E. coli* O157:H7. Displacement of *E. coli* O157:H7 did not occur until after 96 h, whereas in the earlier experiment, using a 50% raw rumen inoculum, displacement was observed after 72 h.

Although we did not determine whether mutants capable of fermenting rhamnose, L-arabinose, and trehalose had emerged, the results we observed with sorbitol may occur with other sugars. Moreover, since some *E. coli* O157 strains ferment sorbitol (10), this approach could not be based solely on sorbitol. However, it is possible that other prebiotic-like nutrients could be identified that could be used to enrich for *E. coli* O157:H7 competitors. That displacement of *E. coli* O157:H7 from the in vitro rumen system can be achieved, as reported here, does suggest that this approach may still be effective as a short-term strategy in reducing carriage of this important pathogen in cattle.

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