Grain Feeding and the Dissemination of Acid-Resistant *Escherichia coli* from Cattle

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The gastric stomach of humans is a barrier to food-borne pathogens, but *Escherichia coli* can survive at pH 2.0 if it is grown under mildly acidic conditions. Cattle are a natural reservoir for pathogenic *E. coli*, and cattle fed mostly grain had lower colonic pH and more acid-resistant *E. coli* than cattle fed only hay. On the basis of numbers and survival after acid shock, cattle that were fed grain had 10^6-fold more acid-resistant *E. coli* than cattle fed hay, but a brief period of hay feeding decreased the acid-resistant count substantially.

Foods can be cooked or irradiated to kill bacteria, but there are ~30 million food-borne illnesses each year in the United States (*1*). A variety of hypotheses have been formulated to explain the increased incidence of food-borne illness (*2, 3*). Modern societies tend to "dine out" more often and consume more processed food. Modern detection methods for pathogenic bacteria are more sensitive, and this sensitivity has heightened our awareness of the problem (*4*). Some experts have suspected a more rapid evolution of bacterial virulence factors, but this evolution is poorly understood (*5*).

Although *Escherichia coli* is a normal inhabitant of the gastrointestinal tract, some strains (for example, O157:H7) produce toxins and are pathogenic (*6*). Hamburger has frequently been contaminated with pathogenic *E. coli*, and vegetables and fruit juices have also been sources of infection (*4*). Cattle, a natural reservoir for pathogenic strains, have often been implicated in *E. coli* infection (*7*). It is virtually impossible to prevent all fecal contamination of meat at slaughter, and vegetables are sometimes fertilized with cattle manure. The ability of *E. coli* to cause food-borne illness is enhanced by its low infective dose, and as few as 10 cells can cause infection (*8*).

The ability of bacteria to act as food-borne pathogens depends on their capacity to survive the low pH of the gastric stomach and to colonize the intestinal tract of humans (*9*), but the role of acid resistance in the dissemination of pathogenic bacteria has often been ignored. Pathogenic and nonpathogenic *E. coli* cultures develop extreme acid resistance only when they are grown at mildly acidic pH. If *E. coli* is grown at neutral pH, it is acid sensitive and killed by the low pH of gastric juice (*10*).

Since the Second World War, fattening beef cattle in the United States have been fed large amounts of grain (starch) and very little hay (*11*), but the impact of grain feeding on acid-resistant *E. coli* had not been examined. Many forms of starch pass through the pregastric stomach (rumen) to the intestines (*11*), and cattle are deficient in the starch-degrading enzyme, amylase (*12*). Starch can be fermented in the colon, and *E. coli* ferments maltose, an extracellular degradation product of starch (*13*). Starch fermentation in the colon produces volatile fatty acids (acetate, butyrate, and propionate) that decrease pH (*12*).

To determine the potential impact of grain feeding on *E. coli* in cattle, we removed colonic digesta from the rectums of cattle that were fed hay, grass, and varying amounts of rolled corn. Digesta were diluted 10-fold with sterile anaerobic water and mixed vigorously with a vortex mixer for 1 min. The pH was measured with a combination electrode. Coliforms were enumerated by visually monitoring turbidity after serial dilution in lauryl sulfate broth (*14*). *Escherichia coli* was determined by screening the bacteria on the basis of lactose fermentation, gas production, indole production, the methyl red reaction, Voges-Proskauer test, and citrate fermentation (*14*). Acid shock was performed by diluting digesta samples 100-fold into Luria broth that had been adjusted to pH 2.0 (*14*). After 1.0 hour at pH 2.0, viable cell numbers were determined by serially diluting into lauryl sulfate broth.

A survey of 61 cattle indicated that grain supplementation could increase total and acid-resistant *E. coli* numbers (Table 1). Cattle fed either hay or fresh grass (pasture) had a colonic pH greater than 7.0, the total *E. coli* count was only 20,000 cells per gram, and virtually all of these bacteria were killed by an acid shock that mimicked the pH of gastric juice. Moderate amounts of grain (60% of dry matter) did not cause a statistically significant decrease in pH ($P < 0.05$), but the total *E. coli* population was 6.3×10^6 viable cells per gram of digesta. Some of the *E. coli* were

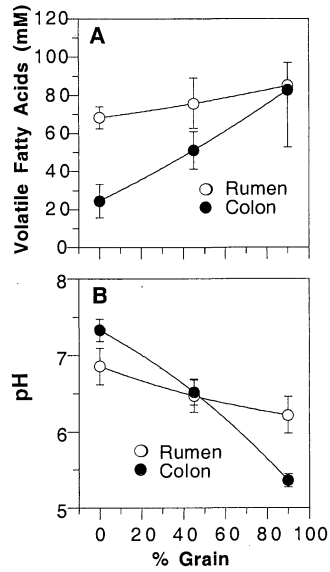

Fig. 1. The effect of grain feeding (percentage of diet dry matter) on (**A**) the volatile fatty acid concentration of the rumen and colon and (**B**) pH. The error bars indicate standard deviations of the mean (three animals, four sampling days).

Table 1. The effect of grain feeding on the colonic pH and *E. coli* counts of cattle fed various amounts of grain.

Diet	Number of animals	Colonic pH	Total *E. coli* (log cells/gram)	Acid-resistant *E. coli** (log cells/gram)
No grain				
Hay	6	7.2 ± 0.1†	4.3 ± 0.5†	<1.0†
Pasture	8	7.1 ± 0.1†	5.0 ± 0.9†	<1.0†
Moderate grain				
60% rolled corn	31	6.9 ± 0.3†	6.8 ± 0.7‡	4.4 ± 1.1‡
Mostly grain				
≥80% rolled corn	16	5.9 ± 0.6‡	6.9 ± 0.9‡	5.4 ± 0.7§

* Acid-resistant *E. coli* are those that survived an acid shock (pH 2.0, Luria broth, 1 hour). †‡§ Means within a column with different superscripts are significantly different ($P < 0.05$, Student's t test).

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killed by acid shock, but the acid-resistant count was greater than 25,000 viable cells per gram. When animals were fed more than 80% grain, the pH was significantly lower ($P < 0.05$), and the acid-resistant *E. coli* count was 250,000 viable cells per gram.

To define more precisely the role of grain in promoting the growth of acid-resistant *E. coli*, we performed highly controlled experiments. Mature, nonlactating Holstein cows were surgically modified so that ruminal contents could be removed directly from the rumen (IACUC protocol 95-1-97). Cattle of similar size (600 kg) were fed every 2 hours with a rotary feeder (10 kg of dry matter per day). The feeds used were medium-quality timothy hay (14% crude protein, 40% neutral detergent fiber) and a grain mixture [89% rolled (cracked) corn and 11% soybean meal]. The diets were 0, 45, and 90% grain with the remainder being hay.

Samples of digesta were obtained from the rumen as well as the colon. Ruminal contents were squeezed through cheesecloth and purged with oxygen-free carbon dioxide. Colonic samples were processed as described above. Samples were centrifuged at 13,000*g* for 10 min to remove bacteria and feed particles, and fermentation acids were analyzed by high-pressure liquid chromatography. The total count of anaerobic bacteria was determined by serially diluting the digesta in a nonselective medium designed for strictly anaerobic bacteria (*15*). Samples of colonic digesta were processed as described above. *E. coli* strains arising from isolated colonies were obtained from MacConkey's plates supplemented with sorbitol as an energy source. *E. coli* strains isolated from cattle and *E. coli* O157:H7 were given an even longer acid shock (6 hours), and in this case the recovery medium was Luria broth.

The randomized block design was a 3 × 3 Latin square (three animals × three diets) with 14 days of adaptation and 4 days of sample collection (total of 54 days). Because the animals were mature, and the environment of the barn was carefully controlled, the effect of time was judged to be inconsequential. The data were first analyzed by two-way analysis of variance (diet versus animal), and the F values indicated that $P < 0.05$ in all cases. Student-Newman-Keuls test (*16*) was used to estimate differences among means, and the variance estimates were pooled (*17*).

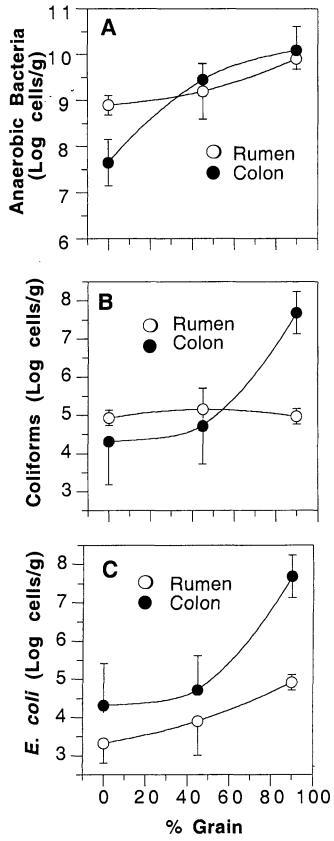

Fig. 2. The effect of grain feeding (percentage of diet dry matter) on (**A**) total anaerobes, (**B**) coliforms, and (**C**) coliforms that were identified as *E. coli*. The error bars indicate standard deviations of the mean (three animals, four sampling days).

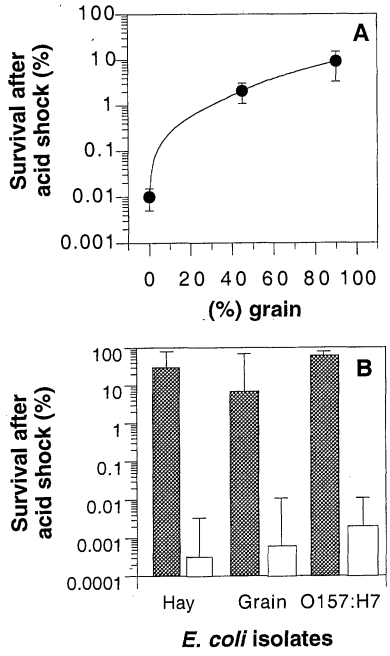

Fig. 3. (**A**) The survival of *E. coli* in colonic fluid (percentage of initial count) after acid shock (pH 2.0, Luria broth, 1 hour), and (**B**) the effect of glucose and final pH on the survival of colonic *E. coli* isolates (hay versus grain) and *E. coli* O157:H7 after acid shock (pH 2.0, Luria broth 6 hours). When the cultures were cultivated overnight in broth containing large amounts of glucose (10 mg solids/ml) (filled bars), the final pH was 4.8. Cultures with small amounts of glucose (0.5 mg/ml) (open columns) produced less acid and the final pH was 6.8. The error bars indicate standard deviations of the mean (10 strains, two replicates per strain).

When cattle were fed increasing amounts of grain, the volatile fatty acid (acetic, propionic, and butyric) concentration of the rumen did not increase significantly ($P > 0.05$), but the concentration in the colon increased ~fourfold ($P < 0.05$) (Fig. 1A). Under these conditions, ruminal pH remained essentially constant ($P > 0.05$), but the pH of the colon decreased ($P < 0.05$) when the volatile fatty acids accumulated (Fig. 1B). Lactic and succinic acids were never detected in rumen samples, but small amounts of both acids were observed in colon samples when 90% grain was fed. Grain supplementation had little effect on the numbers of anaerobic bacteria in the rumen, but the colon count increased 1000-fold (Fig. 2A). Hay-fed cattle had less than 10^5 colonic coliforms, but those fed 90% grain had ~10^8 coliforms per gram of digesta (Fig. 2B). Only a small fraction of the ruminal coliforms were *E. coli*, but virtually all of the colonic coliforms were identified as *E. coli* (Fig. 2C).

Hay-fed cattle had a low concentration of volatile fatty acids in their colons (Fig. 1B), and acid shock killed more than 99.99% of the *E. coli* (Fig. 3A). When diets were supplemented with either 45 or 90% grain, acids accumulated, colonic pH declined (Fig. 1B), and a much larger percentage of the *E. coli* survived acid shock (Fig. 3B). The idea that grain, by promoting acid production in the colon, was regu-

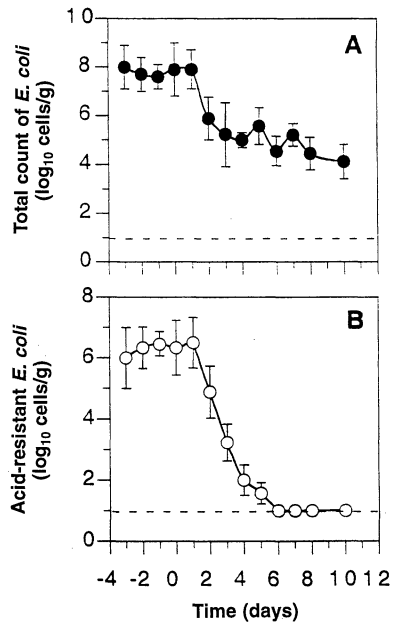

Fig. 4. The effect of hay on the total numbers of colonic *E. coli* in cattle that had been consuming the 90% grain diet. (**A**) Cattle were switched from 90% grain to hay on day zero. (**B**) The numbers of *E. coli* that were able to survival acid shock (pH 2.0, Luria broth, 1 hour). The bars indicate standard deviations of the mean (three animals, one replicate per animal, two independent experiments). The dotted lines show the detection limit of our enumerations.

lating acid resistance in vivo, was corroborated by in vitro experiments. When *E. coli* strains isolated from the cattle were grown in the laboratory with a high concentration of glucose, acetic acid accumulated in the medium, pH declined, and the cell survival after acid shock was high (Fig. 3B). If the glucose concentration of the medium was low, little acid was produced, and cell survival was extremely low. Strains isolated from cattle fed forage or grain, and *E. coli* O157:H7 (ATCC 43895, CDC EDL 933) behaved similarly, and this result indicated that grain feeding was inducing acid resistance rather than selecting a different population of *E. coli*.

About 5% of our *E. coli* isolates ($n = 155$) were sorbitol negative, a diagnostic trait of O157:H7 (*14*), but none of these strains tested positive for O157:H7 antigens (*18*). The absence of *E. coli* O157:H7 in our cattle is not surprising. Previous workers have noted that nonpathogenic *E. coli* can often outgrow pathogenic strains, and this point is illustrated by at least three observations: (i) The percentage of O157:H7-positive animals in herds directly linked to outbreaks was less than 2% (*19*); (ii) even cattle experimentally inoculated with *E. coli* O157:H7 did not shed the bacterium for long periods of time (*20*); and (iii) *E. coli* O157:H7 numbers can be reduced by giving animals doses of nonpathogenic *E. coli* (*21*).

The finding that grain feeding increased both the number and acid resistance of *E. coli* in cattle could have significant implications for food safety. Although not all *E. coli* are pathogenic, there is always the risk that at least some cattle will harbor pathogenic strains. Acid resistance appears to be a factor in the dissemination (transmission) of *E. coli* from cattle to humans. Therefore, it is reasonable to suggest that the induction of acid resistance could increase the risk of foodborne illness. Our studies indicated that the time needed to decrease *E. coli* numbers was relatively short (Fig. 4A). Cattle adapted to a 90% grain diet had an acid-resistant *E. coli* count greater than 10^6 viable cells per gram. After change to a hay diet, the viable cell number immediately declined, and after 5 days the *E. coli* population was nearly 10^6-fold lower (Fig. 4B).

Grain feeding is a practice that promotes the production and efficiency of cattle production, and it is unlikely that American cattle will ever be fed diets consisting only of hay. However, our studies indicate that cattle could be given hay for a brief period immediately before slaughter to significantly reduce the risk of food-borne *E. coli* infection.

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MP1: A MEK Binding Partner That Enhances Enzymatic Activation of the MAP Kinase Cascade

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Signal transduction is controlled both by regulation of enzyme activation and by organization of enzymatic complexes with nonenzymatic adapters, scaffolds, and anchor proteins. The extracellular signal–regulated kinase (ERK) cascade is one of several evolutionarily conserved mitogen-activated protein (MAP) kinase cascades important in the regulation of growth, apoptosis, and differentiation. A two-hybrid screen was conducted to identify nonenzymatic components of this signaling cascade that might be important in regulating its activity. A protein called MP1 (MEK Partner 1) was identified that bound specifically to MEK1 and ERK1 and facilitated their activation. When overexpressed in cultured cells, MP1 enhanced activation of ERK1 and activation of a reporter driven by the transcription factor Elk-1. Expression of MP1 in cells increased binding of ERK1 to MEK1. MP1 apparently functions as an adapter to enhance the efficiency of the MAP kinase cascade.

The MAP kinases ERK1 and ERK2 are components of a protein kinase cascade displaying evolutionary conservation of protein sequence and a three-kinase architecture (*1*). ERKs are activated by the MAP kinase kinases MEK1 or MEK2 . MEKs, in turn, are activated by members of the Raf family. In a related MAP kinase pathway in *Saccharomyces cerevisiae*, the pheromone response pathway, the specificity and efficiency of the enzymatic components are facilitated by a nonenzymatic "scaffolding protein," STE5, that directly interacts with the signaling enzymes of this pathway (*2*).

Although a scaffold-like component has not previously been reported for MAP kinase pathways in higher eukaryotic systems, our earlier work indicated that efficient signaling through the Raf-MEK-ERK pathway also appears to require an additional, unknown component (*3*). MEK1 has a proline-rich sequence (PRS) that spans residues 270 to 307, between kinase subdomains IX and X (*4*). A PRS is present in MEK1 and MEK2, but not in other members of the MAP kinase kinase family. Downstream signaling by MEK requires the PRS; MEK can be activated by mutation to display high enzymatic activity and to transform Rat1 cells, whereas activated MEK mutants that lack the PRS retain

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