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Fates of Acid-Resistant and Non-Acid-Resistant Shiga Toxin-Producing *Escherichia coli* Strains in Ruminant Digestive Contents in the Absence and Presence of Probiotics▽

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Healthy ruminants are the main reservoir of Shiga toxin-producing *Escherichia coli* (STEC). During their transit through the ruminant gastrointestinal tract, STEC encounters a number of acidic environments. As all STEC strains are not equally resistant to acidic conditions, the purpose of this study was to investigate whether acid resistance confers an ecological advantage to STEC strains in ruminant digestive contents and whether acid resistance mechanisms are induced in the rumen compartment. We found that acid-resistant STEC survived at higher rates during prolonged incubation in rumen fluid than acid-sensitive STEC and that they resisted the highly acidic conditions of the abomasum fluid, whereas acid-sensitive strains were killed. However, transit through the rumen contents allowed acid-sensitive strains to survive in the abomasum fluid at levels similar to those of acid-resistant STEC. The acid resistance status of the strains had little influence on STEC growth in jejunal and cecal contents. Supplementation with the probiotic *Saccharomyces cerevisiae* CNCM I-1077 or *Lactobacillus acidophilus* BT-1386 led to killing of all of the strains tested during prolonged incubation in the rumen contents, but it did not have any influence in the other digestive compartments. In addition, *S. cerevisiae* did not limit the induction of acid resistance in the rumen fluid. Our results indicate that the rumen compartment could be a relevant target for intervention strategies that could both limit STEC survival and eliminate induction of acid resistance mechanisms in order to decrease the number of viable STEC cells reaching the hindgut and thus STEC shedding and food contamination.

Shiga toxin-producing *Escherichia coli* (STEC) strains are food-borne pathogens that cause human diseases ranging from uncomplicated diarrhea to hemorrhagic colitis (HC), as well as life-threatening complications, such as hemolytic-uremic syndrome (HUS). Most outbreaks and sporadic cases of HC and HUS have been attributed to O157:H7 STEC (http://www.cdc.gov/ecoli/outbreaks.html; http://www.euro.who.int). However, in some geographic areas, non-O157:H7 STEC infections are considered to be at least as important as *E. coli* O157:H7 infections, but they are often underdiagnosed (21, 46). In spite of diverse virulence characteristics, one common trait of pathogenic STEC strains could be resistance to the gastric acidity in humans. Indeed, it has been suggested that acid resistance of *E. coli* O157:H7 is negatively correlated with the infectious dose required for this organism to cause disease in humans (17).

Healthy cattle and other ruminants appear to be the main reservoir of STEC strains. However, colonization of the cattle gastrointestinal tract (GIT) by STEC seems to be a transient event, with a mean duration of 14 days to 1 month (4, 8, 38). The site of STEC persistence and proliferation in the GIT depends on the STEC strain and seems to vary from one individual to another. Some previous studies identified the rumen as the primary site of colonization (8), whereas other studies referred to the cecum, the colon, or the rectum (10, 18, 23, 32, 42). Although STEC strains adhere *in vitro* to bovine colonic mucosa, forming the characteristic attaching and effacing lesions (35), they are very rarely associated with tissues in animal carriers and are generally isolated from the digesta (8). STEC does not, therefore, seem to colonize the gut mucosa, except for the anorectal mucosa, which has been described as the preferred colonization site for O157:H7 strains but not for non-O157:H7 strains (24, 32). During their transit through the ruminant GIT, STEC strains encounter various acidic conditions. Volatile fatty acid (VFA) concentrations are high in the rumen of grain-fed animals, and the pH may vary from 5.0 to 6.5. In these conditions, VFAs are in the undissociated form and can freely enter the bacterial cells, dissociate, and acidify the cytosol. In hay-fed animals, less fermentation occurs in the rumen, and the pH remains between 6.5 and 7. In the abomasum, STEC encounters strongly acidic conditions, regardless of the diet, due to the presence of mineral acids, resulting in a pH below 3. Then the pH increases from the proximal part to the distal part of the small intestine, and in the cecum and the colon STEC encounters more neutral pH conditions.

All STEC strains are not equally resistant to acidic conditions (2, 9, 30, 45). Therefore, it could be hypothesized that

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TABLE 1. Virulence factors and acid resistance of STEC strains

Strain	Serotype	Origin	Presence of[a]:			Shiga toxin production[b]	% Survival after acid challenge for[c]:		Reference
			eae	stx_1	stx_2		2 h	20 h	
EDL933	O157:H7	Meat	P	P	P	P	56	0	33
ANR 245A1	Ont:H8	Raw milk cheese	N	P	P	P	97	41	43
ANR V1	O166:H28	Environment	N	N	P	P	94	44	44
ANR V10	O11:H43	Environment	N	P	N	P	83	46	44
ANR 415A	O6:H10	Raw milk cheese	N	P	P	P	85	47	43
ANR 42A	O6:H1	Raw milk cheese	N	P	P	P	3	0	43
ANR 418A	O6:H10	Raw milk cheese	N	P	P	P	14	0	43
ANR 346A	O174:H8	Raw milk cheese	P	P	P	P	28	0	43
ANR 360B	O6:H1	Raw milk cheese	N	P	P	P	4	0	43

[a] P, positive as determined by PCR; N, negative as determined by PCR.
[b] P, Shiga toxin produced as determined by verocytotoxicity.
[c] The acid challenge experiment was performed as described in Materials and Methods.

acid-resistant (AR) STEC survives and persists better in the GIT of ruminants than acid-sensitive (AS) STEC. Acid resistance mechanisms can be induced during exposure to a moderately acidic environment (12, 26, 41). The rumen contents of a grain-fed animal could be such an environment favorable for the induction of acid resistance in STEC. While the diet does not seem to affect the acid resistance of an *E. coli* O157:H7 strain (19), grain feeding increases the number of acid-resistant generic coliforms (15, 19), either by inducing acid resistance mechanisms in the rumen or by selecting acid-resistant *E. coli* strains during passage through the abomasum. Hence, generic coliforms behave differently than *E. coli* O157:H7 in ruminants (19), and the potential ecological advantage conferred by acid resistance to non-O157:H7 STEC strains for persistence in the ruminant GIT has never been investigated.

Inhibition of STEC proliferation in the ruminant gut may be mediated through probiotic supplementation. Several studies have demonstrated the capacity of certain lactic acid bacteria or yeast to reduce *E. coli* O157:H7 counts *in vitro* (1, 34) or *in vivo* (5, 40). The mechanisms of action of probiotics are not well characterized but could involve competition for nutrients and adhesion sites in the GIT, an increase in the VFA concentration and a decrease in the pH, production of antimicrobial molecules, or interference with quorum-sensing signaling (27–29). However, the impact of probiotics on non-O157:H7 STEC has been poorly investigated (36). Although not all non-O157:H7 STEC strains are pathogenic, limiting their carriage by ruminants should decrease the risk of food-borne illness. The impact of probiotics and of the physicochemical conditions of the rumen digesta on the survival of non-O157:H7 STEC strains or on induction of acid resistance mechanisms could have significant implications for farm management practices and food safety.

The purpose of this work was to investigate whether the level of acid resistance, determined using an *in vitro* assay, confers an ecological advantage to STEC strains in ruminant digestive contents and whether acid resistance mechanisms are induced in the rumen compartment. Moreover, we evaluated the potential of probiotics to limit STEC survival and induction of acid resistance in the ruminant GIT.

MATERIALS AND METHODS

STEC strains. The AR and AS non-O157:H7 STEC strains used in this study (Table 1) have been described previously (43, 44) and were used to examine whether acid resistance conferred a growth or survival advantage during manufacture of fermented raw meat sausages and raw milk Camembert cheeses (30, 31). *E. coli* O157:H7 strain EDL933 was isolated from ground meat during an outbreak in the United States (33). This strain was shown to be AR after a 2-h acid challenge but AS after a 20-h acid challenge (Table 1). Spontaneous rifampin-resistant mutants were selected for each strain. Neither the growth rate nor the acid resistance properties of the rifampin-resistant derivatives were affected when the organisms were tested using the AR assay described below.

AR assay. Single colonies were grown for 8 h in Luria-Bertani broth (LB). The cultures were diluted 1:500 into LB buffered with 100 mM morpholinethanesulfonic acid (MES) (pH 5) and grown overnight, and then they were diluted 1:1,000 in M9 medium (pH 2.5) supplemented with 0.4% (wt/vol) glucose and 0.4% (wt/vol) Casamino Acids. The percentage of survival was calculated from viable cell counts at 0, 2, and 20 h after the acid challenge. Three independent experiments were performed for each strain (Table 1).

Origin and preparation of digestive samples. Animals used in this study were housed in individual stalls in experimental facilities of the Unit of Research on Herbivores at INRA Research Centre, Theix, France, in accordance with the guidelines of the local ethics committee. Nine rumen-cannulated sheep were used for collection of digestive samples. They were first fed meadow hay (H diet) for 2 weeks, and then, after a transition period of 10 days, they received a mixed diet containing hay (40% on a dry matter basis) and pelleted ground wheat (60%) for 4 weeks (HW diet). The diet (1 to 1.1 kg dry matter/day) was distributed in two equal meals at 8:00 a.m. and 16:00 p.m. Three of these nine sheep received through the cannula 4×10^9 CFU of *Saccharomyces cerevisiae* CNCM I-1077 per day, and three other sheep received 5×10^9 CFU of *Lactobacillus acidophilus* BT-1386 per day. The remaining three sheep did not receive any probiotic supplement and were used as controls. Both probiotic strains were supplied by Lallemand Animal Nutrition, Blagnac, France. During the HW diet feeding period, rumen samples were collected once a week from the midventral sac 3 h after the morning feeding. These samples were processed under strictly anaerobic conditions as previously described (13), with minor modifications. Briefly, rumen digesta were collected in O_2-free N_2-saturated sterile flasks. Samples were immediately brought to the laboratory, where they were strained through four layers of cheesecloth. Samples from sheep in the same group were pooled using equal proportions in N_2-saturated sterile flasks closed with rubber stoppers and sealed with aluminum caps. The pools prepared for each day of sampling were immediately frozen at −80°C. We previously checked that these conditions were an appropriate way to maintain the viability of the rumen microbiota. Samples from other parts of the sheep gastrointestinal tract were collected at slaughter. For each animal, we obtained samples from the abomasum, jejunum, and cecum. The samples were collected in sterile flasks previously filled with N_2. Pools from each digestive compartment were composited for the three groups of sheep and were immediately frozen at −80°C. An aliquot of each pool was used for pH measurement. Other aliquots were kept and used for

TABLE 2. Concentrations of fermentation products before and after incubation of rumen, jejunal, and cecal samples with and without probiotics and without STEC strains

Digestive sample	Probiotic	Incubation time (h)	pH	Concn (mM) of[a]:		
				Acetate + propionate + butyrate	DL-Lactate	Ethanol
Rumen contents	None	0	5.65	93.3 ± 4.8	—	2.4
		24	4.96	146.5 ± 2.4	30.2	6.6 ± 2.1
	S. cerevisiae	0	5.87	106.8 ± 13.1	5.5 ± 1.9	3.4 ± 0.2
		24	4.67	156.8 ± 19.1	26.9 ± 0.7	58.9 ± 2.9
	L. acidophilus	0	5.65	110.2 ± 2.5	7.7 ± 1.3	5.5 ± 4.3
		24	5.42	133.7 ± 5.2	55.0 ± 0.3	7.7
Jejunal contents	None	0	7.82	—	—	—
		2	7.68	3.0 ± 0.2	—	8.8 ± 2.2
	S. cerevisiae	0	7.32	4.7 ± 0.1	—	25.4 ± 0.8
		2	7.38	23.1 ± 1.5	—	27.9 ± 0.3
	L. acidophilus	0	7.82	2.3 ± 0.1	15.02	4.5 ± 0.1
		2	7.13	14.2 ± 2.4	19.5	8.8 ± 0.3
Cecal contents	None	0	7.72	31.5 ± 0.7	—	4.4 ± 0.4
		8	7.73	39.0	—	4.1 ± 0.1
	S. cerevisiae	0	7.72	29.8 ± 0.5	3.5 ± 1.9	—
		8	7.70	40.3 ± 0.2	8.2 ± 1.2	4.7 ± 0.6
	L. acidophilus	0	7.60	29.9 ± 0.1	ND	3.8 ± 0.1
		8	7.68	33.1 ± 0.1	ND	3.7 ± 0.1

[a] The values are means ± standard errors of the means for at least three determinations. —, not detected; ND, not done.

biochemical analyses. The remaining aliquots were used as culture media to examine the behavior of the AR or AS STEC strains.

Incubation of STEC strains in the digestive contents. The rifampin-resistant derivatives of the nine STEC strains were incubated individually in the digestive contents of sheep fed the HW diet with and without probiotics under strictly anaerobic conditions (N_2-saturated atmosphere) with gentle shaking (rumen and cecum contents) or in a normal atmosphere with no shaking (abomasal and intestinal contents) at 39°C for 6 or 24 h in the rumen fluid, for 1 or 4 h in the abomasum contents, for 2 h in the jejunal contents, and for 8 h in the cecal contents. These conditions (temperature, oxygenation, and duration) were chosen to reflect the *in vivo* conditions for each digestive compartment. For the rumen samples, ground feed (40% hay, 60% wheat) was added (25 mg of mixed ground feed for 5 ml of rumen contents) to mimic a fermentation cycle, as previously described (13). Because probiotics need to be administered daily, they were added to the incubation tubes containing the digestive fluids from animals fed probiotics as previously described (13) to obtain a final concentration close to 3×10^5 cells ml^{-1} of digestive contents. LB supplemented with rifampin (100 $\mu g\ ml^{-1}$) was inoculated with each of the STEC strains and incubated overnight at 37°C with shaking. The optical densities of these precultures were adjusted to inoculate the tubes containing the digestive contents with $\sim 5 \times 10^5$ cells ml^{-1} of each STEC strain separately. The precise concentrations of viable STEC cells at the start and at the end of the incubation were determined by enumeration after serial 10-fold dilution in phosphate-buffered saline on solid LB medium containing 100 $\mu g\ ml^{-1}$ rifampin. Each experiment was done independently at least three times.

Survival of STEC strains in the abomasal fluid after incubation in the rumen contents. Selected rifampin-resistant STEC strains were incubated individually for 6 h in the rumen contents as described above. Bacteria were pelleted, and the abomasum fluid was inoculated with $\sim 5 \times 10^5$ STEC cells ml^{-1} and incubated for up to 4 h at 39°C. Survival was calculated by enumeration of the STEC at the beginning of the incubation and at different time points. STEC strains were also incubated in the abomasum fluid in the same conditions but without preincubation in the rumen contents. Each experiment was done independently at least three times.

Analysis of fermentation products in the digestive contents. Before and after incubation, total volatile fatty acid (VFA) concentrations in digestive samples were determined by gas chromatography. Ethanol and DL-lactate concentrations were determined with enzymatic assays (SCIL Diagnostics, Germany).

Statistical analyses. Analysis of variance was used to assess the significance of the results. A P value of <0.05 was considered significant.

RESULTS

Growth of AR and AS STEC in sheep digestive contents. To investigate whether acid resistance conferred a growth advantage to STEC strains in the different compartments of the ruminant GIT, we incubated each strain in rumen, abomasum, jejunum, and cecum contents of animals fed the HW diet. To mimic the physiological duration of transit through the GIT and the oxygen availability, the preparations were incubated for 24 h under strictly anaerobic conditions for the rumen fluid and without aeration for 1.5 h for the abomasum fluid, for 2 h for the jejunal contents, and for 8 h for the cecal contents.

The pH of the rumen contents was 5.65, and it decreased (by 0.69 pH unit) in the control tubes that did not contain STEC after 24 h of incubation due to the fermentative activities of the rumen microbiota, as shown by the high level of volatile fatty acids (Table 2). After incubation of the STEC strains, the mean pH was 4.56 ± 0.05 (mean decrease, 1.09 pH units). The four AR non-O157:H7 strains did not grow but had survival rates between 17 and 38% (Fig. 1A). Two AS STEC strains had survival rates of 34 and 10%, which were similar to those of AR strains, whereas the two other AS STEC strains did not survive. Statistical analysis indicated that the average survival rate of AR STEC was significantly higher than the average survival rate of AS STEC ($P < 0.01$). Surprisingly, the O157:H7 strain did not survive, whereas it appeared to be AR after a 2-h acid challenge. However, this strain was not resistant to a 20-h acid challenge, in contrast to the non-O157:H7 AR strains (Table 1). A kinetic analysis of one AR strain, two AS strains, and EDL933 indicated that all these strains survived for at least 11 h in the rumen fluid, and then the viable counts began to decrease (Fig. 1B).

In the abomasum fluid (pH 2.5) the survival rates of AR

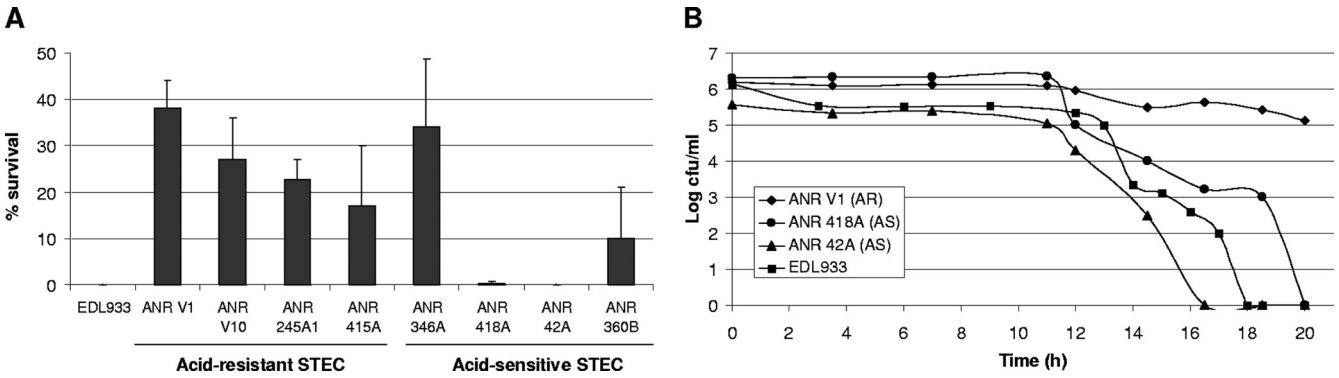

FIG. 1. Survival of STEC strains in the rumen fluid. Three independent experiments were performed. (A) STEC survival rates after 24 h of incubation. (B) STEC survival curves. A representative curve is shown for each strain.

strains, including the O157 and non-O157 strains, varied from 14.5 to 58% (Fig. 2), whereas AS strains did not survive (survival rates, 0 to 0.07%) (data not shown).

The pH of the jejunal and cecal contents remained near 7.7 during incubation, due to lower fermentative activities of the resident microbiota (Table 2) and probably due to the neutralizing effect of bicarbonate. The AR and AS strains proliferated (Fig. 3) in the jejunal and cecal contents. The growth of AR STEC (mean, 1.09 log) was slightly but significantly ($P < 0.01$) greater than the growth of AS STEC (mean, 0.68 log) in the jejunal contents, whereas the acid resistance status had no influence on STEC growth in the cecal contents.

Induction of acid resistance mechanisms in the rumen fluid. We wanted to investigate whether the acidic stress conditions of the rumen contents induced STEC acid resistance and promoted AS STEC survival in the highly acidic abomasum fluid. Selected STEC strains were incubated for 6 h in the rumen fluid (pH 5.65, 93.3 mM VFAs) before they were challenged in the abomasum fluid. As shown in Fig. 4A, non-O157 and O157 AR strains showed a high survival rate in the abomasum contents up to 4 h, and preincubation in the rumen contents did not have any influence on their survival in the abomasum fluid. In contrast, the survival rate of the AS STEC strains increased greatly after incubation in the rumen fluid. As a control, we performed the same experiment with the preincubation step using rumen fluid samples from sheep fed 100% hay that were collected before feeding. This rumen fluid had a higher pH (pH 6.93) and a slightly lower concentrations of VFAs (84.5 mM) than rumen fluid from sheep fed the HW diet. At this pH, VFAs are in the dissociated form and cannot enter bacterial cells. Surprisingly, preincubation of the AS STEC strains in this rumen fluid also increased the capacity of the strains to resist the acid challenge in the abomasum (Fig. 4B), indicating that induction of acid resistance mechanisms was not mediated (or not entirely mediated) by the combination of moderately acidic pH and high VFA concentrations found in the rumen contents of grain-fed sheep.

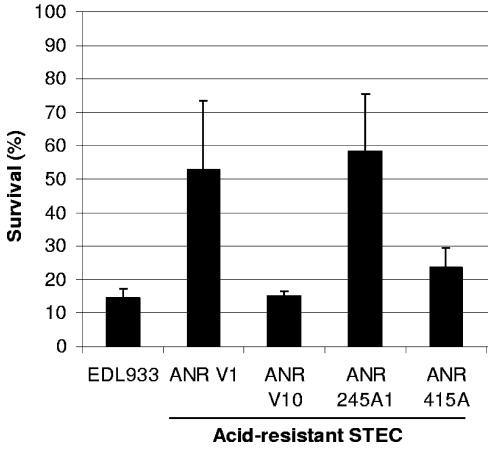

FIG. 2. STEC survival rates after 1.5 h of incubation in the abomasal fluid. Three independent experiments were performed.

Influence of probiotics on STEC survival in the sheep digestive contents. STEC strains were incubated in the digestive contents of sheep fed the HW diet supplemented with the probiotic *S. cerevisiae* I-1077 or *L. acidophilus* BT-1386. The pH of the rumen contents of sheep fed *S. cerevisiae* was slightly higher than the pH of the rumen contents of sheep fed *L. acidophilus* (pH 5.87 versus pH 5.65), and the values dropped to pH 4.68 and 4.38, respectively, after 24 h of incubation with the STEC strains, while the survival rate of STEC varied between 1.48 and 0%. Very low levels of STEC cells were recovered for five STEC strains (four AR strains and one AS strain) in the presence of *S. cerevisiae*, but STEC cells were never recovered in the presence of *L. acidophilus*, even after a culture enrichment step. In the rumen contents containing *S. cerevisiae*, high concentrations of ethanol were found (Table 2), and in the samples containing *L. acidophilus*, the lactate concentration was increased, whereas the concentrations of the three major VFAs (acetate, propionate, and butyrate) remained close to those in samples without probiotics.

In contrast to the results for rumen contents, probiotic supplementation did not inhibit STEC growth in the jejunal and cecal contents (data not shown). In jejunal samples there was an increase in the concentration of major VFAs in the presence of probiotics (Table 2), which remained less than 25 mM. The concentration of lactate in *L. acidophilus* incubations was also higher than the concentration in the absence of probiotics, but it remained less than 20 mM. In cecal contents, no change in the concentration of fermentation products was seen in the presence of probiotics.

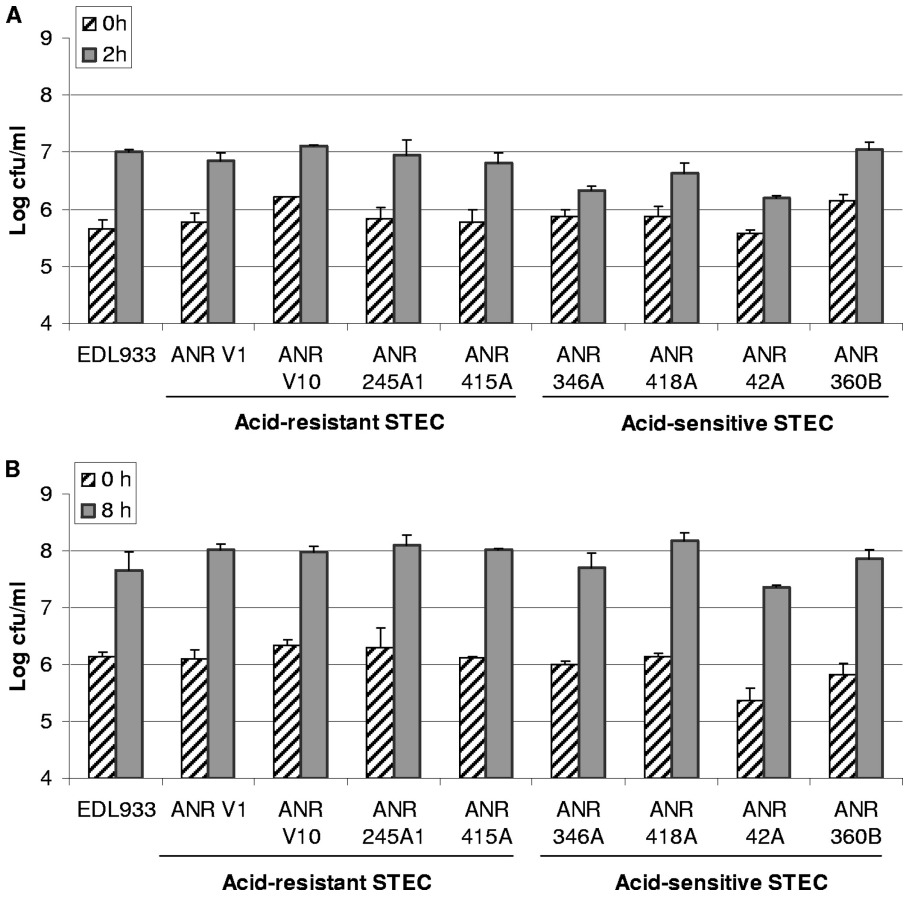

FIG. 3. Growth of STEC strains in jejunal (A) and cecal (B) contents. Three independent experiments were performed.

Since *E. coli* O157:H7 did not survive for 24 h during incubation in the rumen fluid but survived for at least 12 h, we performed a growth kinetic analysis in the presence of probiotics. As described above, the O157:H7 strain survived for about 12 h in the rumen fluid, and then the concentration of viable cells began to decrease (Fig. 5). No viable cells were detected after 18 h. In the presence of *L. acidophilus*, the cell concentration decreased more rapidly, and no viable cells were

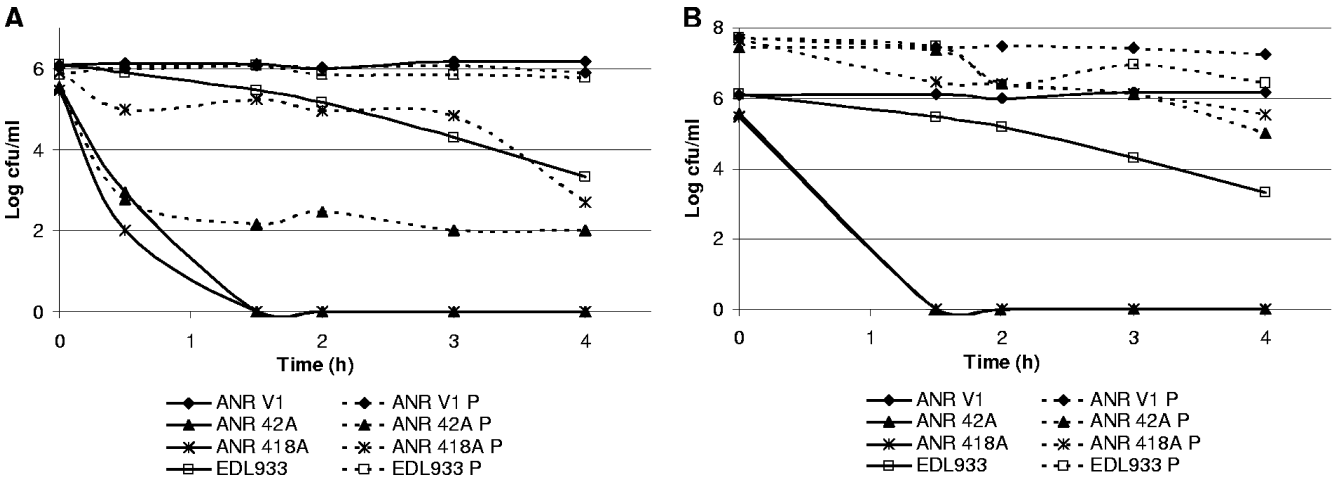

FIG. 4. Influence of 6 h of preincubation in rumen contents on the survival of STEC strains in the abomasum fluid. Solid lines and dotted lines indicate the survival of STEC in the abomasum without preincubation and after preincubation (P), respectively. (A) Preincubation in the rumen contents of grain- and hay-fed animals. (B) Preincubation in the rumen contents of hay-fed animals. Three independent experiments were performed. A representative curve is shown for each strain.

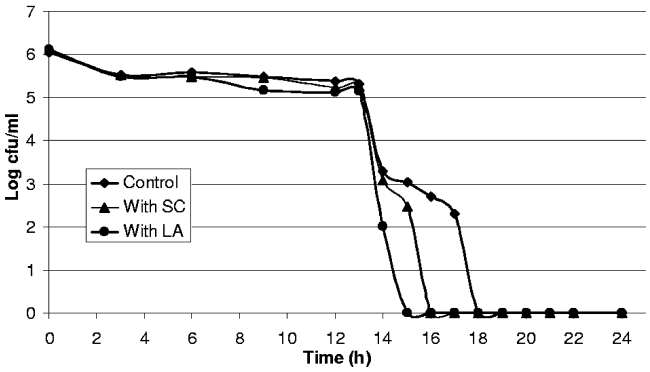

FIG. 5. Effect of probiotics on EDL933 survival in the rumen fluid. Control, EDL933 survival without probiotics; SC, EDL933 survival in the rumen fluid in the presence of *S. cerevisiae*; LA, EDL933 survival in the rumen fluid in the presence of *L. acidophilus*. Three independent experiments were performed. A representative curve is shown for each condition.

detected after 15 h of incubation; in the presence of *S. cerevisiae* no viable cells were detected after 16 h.

To determine whether probiotic yeast could help alleviate the induction of acid resistance mechanisms in the rumen fluid, we preincubated the STEC strains in rumen fluid harvested from animals fed the probiotic yeast before the acid challenge. We observed that the acid resistance mechanisms were still induced (data not shown).

DISCUSSION

It has been proposed that acid-resistant STEC strains colonize other animals and possibly humans more efficiently than acid-sensitive strains, since they may survive better in the acidic compartments of the GIT (rumen, abomasum, or stomach). Acid resistance may also promote STEC survival in acidic food and thus represent a risk factor for food-borne infections. The ability of serotype O157:H7 STEC isolates to survive in the bovine GIT and to colonize cattle, as well as their levels of acid resistance before and after transit through the animals, has been investigated (13, 19, 20), but little is known about non-O157 serogroups. In particular, survival of non-O157:H7 strains in the bovine GIT in relation to their acid resistance status has not been described previously. In this study, we found that acid resistance may confer an ecological advantage to non-O157:H7 STEC in the rumen digesta, although two of the four acid-sensitive strains tested survived at the same level as the acid-resistant strains. In the rumen fluid the reference O157:H7 strain EDL933, which was responsible for a large outbreak in the United States, behaved like acid-sensitive non-O157:H7 strains. A larger number of strains should be tested to confirm the better survival of AR strains in the rumen fluid and to evaluate whether the *in vitro* AR assay is the most relevant way to characterize acid resistance status in the GIT. Indeed, the higher levels of survival of some strains in the rumen contents could be unrelated to their acid resistance status but could depend on resistance to other factors (for example, resistance to antimicrobials produced by the resident microbiota or to defensins produced by the host). All of the strains tested survived in the rumen fluid for up to 10 h regardless of their acid resistance status. After 10 h, the counts of the acid-sensitive strains began to decrease dramatically. Hence, acid resistance promotes STEC survival in the rumen contents only when there is prolonged retention in this digestive compartment. The mean retention time of STEC in the rumen is not known. It probably depends on the association of STEC cells with the liquid phase or with the solid phase. Brossard et al. (7) determined that the turnover time of the liquid phase is 5 to 7 h. Therefore, if STEC cells are associated with the liquid phase, they should survive at the same level regardless of their acid resistance. In the case of the association of STEC cells with the solid phase of the rumen digesta, the longer turnover time (more than 20 h) leads to prolonged retention of the strains in the rumen compartment, resulting in mortality of only the AS STEC strains. We observed that the transit of STEC strains in the rumen for 6 h promoted induction of acid resistance mechanisms independent of the diet, allowing AS STEC strains to survive in the highly acidic environment of the abomasum at the same level as AR strains. This observation is in agreement with *in vivo* studies showing that the acid resistance of *E. coli* O157:H7 is not affected by diet (19, 20), and it extends this conclusion to non-O157:H7 strains. In contrast, generic coliforms from hay-fed animals have been shown to be more sensitive to an acid shock than coliforms from grain-fed animals (15, 19). Our data indicate that an STEC strain grown *in vitro* that is observed to be acid sensitive according to a classical *in vitro* assay probably becomes acid resistant after its transit through the ruminant GIT. This rules out acid resistance status as a criterion to estimate the risk factor associated with this strain. A question arising from our data is the nature of the environmental cues that induce the acid resistance mechanisms in the rumen digesta independent of the pH. More experiments should be done to resolve this question.

In this study we observed significant growth of all the STEC strains in jejunal and cecal contents. The ecological conditions encountered in these contents were favorable for bacterial growth, because of the stable pH, the possible presence of oxygen, the low levels of organic acids, and a microbial environment that was less competitive than that in the rumen. We did not identify the nature of the carbon and nitrogen sources used by the STEC strains for growth. Fabich et al. (16) reported that O157:H7 strains are able to take up specific sugars present in the mucus layer of the mouse intestine, suggesting that invading pathogens gain an advantage by consuming several sugars that may be available because they are not consumed by the commensal intestinal microbiota. We have found that ethanolamine, a compound present in the bovine intestine, can be used by *E. coli* O157:H7 as a nitrogen source, and a transcriptomic approach has indicated that several metabolic pathways involved in utilization of mucus-associated sugars were upregulated when *E. coli* O157:H7 was grown in bovine gut contents (3, 25).

The acidic conditions in the GIT are not the sole factor influencing STEC survival. The resident digestive microbial populations produce a number of antimicrobials that influence STEC survival independent of the pH. For example, *E. coli* strains isolated from cattle produce colicins and show potential to displace *E. coli* O157:H7 in live cattle (48), and Brashears et al. isolated numerous lactic acid bacteria from healthy cattle

that limit *E. coli* O157:H7 survival in rumen fluid with no change in the pH (6). Therefore, probiotic supplementation has been proposed as a way to reduce *E. coli* O157:H7 shedding by ruminants (39). In the current experiments we found that *L. acidophilus* and *S. cerevisiae* significantly reduce O157 and non-O157 STEC counts in the rumen fluid. These effects were observed to be independent of the acid resistance of the strains. The mechanisms of action of probiotics have not been clearly identified. We found high ethanol concentrations in *S. cerevisiae* I-1077-supplemented incubations, and use of ethanol has been reported to be a possible way to eliminate various food-borne pathogens from vegetable surfaces (47) or to reduce or eliminate *E. coli* O157 strains in other situations, especially in low-pH conditions (22). *S. cerevisiae* I-1077 has also been shown to affect the growth and activities of rumen microbial communities (14), so STEC inhibition could also be associated with a modified balance of the resident microbiota. *L. acidophilus* BT-1386 has been reported to limit the prevalence of O157:H7 in naturally infected feedlot cattle (40), and it inhibits the *in vitro* growth of *E. coli* O157:H7 in fecal suspensions (13). In this study, we found high concentrations of lactate in the rumen contents incubated with *L. acidophilus*, which presumably led to the observed decrease in pH. The accumulation of lactate and the subsequent decrease in the pH could explain the complete eradication of all STEC strains tested. In several studies lactic acid bacteria have been reported to exhibit inhibitory activity against *E. coli* O157, either by limiting bacterial growth or by inhibiting stx_2 gene expression through production of organic acids, a decrease in the pH, or production of inhibitory molecules (11, 28, 34). However, a recent study described the growth-promoting effect of certain strains of lactic acid bacteria on pathogenic *E. coli* strains (37).

In our *ex vivo* assays, the metabolites produced by probiotics accumulated. Thus, lactic acid and ethanol concentrations could be different *in vivo* due to reabsorption by the rumen epithelium. Quantification of these metabolites in animals fed the supplemented diet would allow further analysis of the efficacy of probiotics.

Our *ex vivo* model of STEC infection allows monitoring of STEC strain behavior in each digestive compartment of the bovine GIT. We found that STEC mortality occurs in the rumen and abomasum fluids, whereas all STEC strains actively grow in the jejunal and cecal contents, independent of their acid resistance. Consequently, preharvest strategies that effectively limit STEC growth in the cattle GIT should target the intestine. However, the efficacy of these strategies would be increased if only a limited number of STEC cells were able to reach the hindgut. In this respect, the rumen might be a relevant target not only to maximally reduce the number of viable STEC cells that can be transferred to the hindgut but also to inhibit induction of acid resistance mechanisms, limiting the risk associated with acid-resistant strains for the food industry. The probiotic yeast and lactic acid bacteria used in this study dramatically reduced STEC survival in the rumen digesta, but they had no influence on acid resistance induction or on STEC growth in the hindgut compartments. Furthermore, the decrease in STEC cell counts in the rumen fluid occurred only after 15 to 16 h of incubation, and the acid resistance mechanisms are induced within 6 h. Therefore, it is crucial to find probiotic strains that are able to inhibit STEC survival in the rumen fluid more rapidly. Higher probiotic concentrations, mixtures of probiotic strains with other inhibitory compounds, or synbiotics consisting of combinations of selected strains together with their specific substrates may be interesting ways to improve probiotic efficacy in the rumen.

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