Effect of diet on shiga toxin-producting Escherichia coli (STEC) growth and survival in rumen and abomasum fluids

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Original article

Effect of diet on Shiga toxin-producing *Escherichia coli* (STEC) growth and survival in rumen and abomasum fluids

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Abstract – The gastrointestinal tract of ruminants is the main reservoir for Shiga toxin-producing *Escherichia coli* (STEC) strains, potentially pathogenic for humans. We used for the first time rumen fluid in which no exogenous carbon source or other supplement was added to compare acid resistance and growth of STEC in physiological physico-chemical conditions. We showed that acidic conditions resulting from the combination of high volatile fatty acid concentration and moderately acidic pH did not alter the survival of STEC, and that human non-O157:H7 STEC isolates were able to persist in the rumen contents in spite of acid stress, low oxygen availability and nutrient deprivation, in the same manner as bovine STEC isolates do. Furthermore, our results support the hypothesis that a grain-rich diet may induce mechanisms of STEC acid resistance in the rumen that allow STEC survival in the abomasum.

STEC / non-O157:H7 / rumen / acid-resistance

Résumé – **Effet du régime alimentaire sur la survie et la prolifération des *Escherichia coli* producteurs de Shiga-toxines (STEC) dans du jus de rumen et de caillette.** Le tube digestif des ruminants est le principal réservoir de souches *d'Escherichia coli* producteurs de Shiga-toxines (STEC) potentiellement pathogènes pour l'Homme. Nous avons utilisé pour la première fois du jus de rumen non dilué dans lequel aucune source de carbone exogène ni aucun supplément nutritionnel n'a été ajouté afin d'étudier la résistance à l'acidité et le devenir des STEC dans des conditions physico-chimiques naturelles. Nous avons montré que les conditions acides résultant de la combinaison de fortes concentrations d'acides gras volatils et d'un pH modérément faible (6.0) ne diminuaient pas la

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survie des STEC, et que les isolats humains non O157:H7 étaient capables de persister dans le jus de rumen, en dépit de la faible disponibilité en oxygène et en nutriments, de la même façon que des isolats bovins. De plus, nos résultats supportent l'hypothèse selon laquelle un régime riche en grain induit chez les STEC la mise en place d'un mécanisme de résistance au stress acide dans le rumen, ce qui leur permet ensuite de mieux résister au passage dans la caillette.

STEC / non-O157:H7 / rumen / stress acide

1. INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) are important food-borne pathogens causing bloody diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans. Shiga toxins Stx1 and Stx2 are the virulence factors responsible for the hemorrhagic symptoms of the disease [9]. Ruminants are thought to be the principal reservoir for Shiga toxin-producing *E. coli*, which is potentially pathogenic for humans. Although O157:H7 *E. coli* is the main causative agent of the hemolytic uremic syndrome (HUS) due to STEC outbreaks, more than 100 other serotypes have been associated with sporadic cases of human disease, most of them also being isolated from ruminants [13]. Thus, decreasing the carriage of STEC strains by ruminants would help to reduce the risk of food-borne disease in humans.

In animals experimentally infected with high doses of O157:H7 *E. coli*, it appears that hay-fed sheep or cattle shed the organism for longer periods than grain-fed animals [5, 7]. However, hay-rich diets have been reported to clear O157:H7 *E. coli* completely from the gastrointestinal (GI) tract, in contrast to grain-rich diets [6]. In all studies, the small number of animals used and the high individual variations observed contributed to poor statistical significance and obvious conclusions were difficult to draw. Thus in vitro studies seem necessary for a better understanding of STEC ecology and physiology in the conditions of the ruminant GI tract. The very few studies investigating STEC growth in rumen fluid concerned only the O157:H7 strains and always used rumen fluid in which glucose or trypticase was added [11]. However, only trace amounts of hexoses are available in vivo for *E. coli* in the rumen fluid since hexoses are rapidly assimilated by the dominant flora.

Studies addressing the possible induction of *E. coli* acid-resistance in the GI tract of ruminants fed hay or grain are also controversial [5, 12]. This question is crucial since acid-resistant *E. coli* will be more likely to survive in acidic food and in the GI tract of humans. The number of bacteria that reach the gut is directly dependent on the number of bacteria that resist the acid shock in the abomasum where the mean pH is typically 2.5. Induction of STEC acid resistance has never been tested in the physiological conditions of pure rumen fluid and abomasum fluid. Thus it is of major importance to determine whether or not the environmental conditions to which bacteria are subject in the rumen influence their survival in the acidic environment of the abomasum.

In the present study, we investigated the behaviour of clinical and bovine STEC isolates, including non-O157:H7 serotypes, in undiluted, unsupplemented rumen fluids. This study focused on the influence of abiotic factors by using sterile rumen fluids. We showed that STEC were able to proliferate in aerobiosis in rumen fluids using acetate as the carbon source, and to survive in anaerobiosis without a loss of viability. A diet rich in grain leads to an acidic rumen fluid that did not support STEC growth even in aerobiosis, but induced STEC acid

resistance, allowing bacterial survival during the passage through the abomasum.

2. MATERIALS AND METHODS

2.1. Collection and analysis of rumen fluid

Two sheep fitted with rumen fistulas and housed separately in concrete stalls were fed twice a day with a diet containing 100% dactyl hay for three weeks, then 50% dactyl hay/50% corn for three more weeks. A volume of 1.5 to 2 litres of rumen fluid was collected through the fistulas during each of the last five days of each diet from sheep fasting in the morning (18 hours post-feeding) and 90 min after feeding. In this manner, four pools of rumen fluid were obtained: R1 and R2 from sheep fed with 100% hay, collected 18 hours and 90 min after feeding, respectively; R3 and R4 from sheep fed 50% hay/50% corn, collected 18 hours and 90 min after feeding, respectively. Each rumen pool was gauze-filtered, centrifuged twice for 30 min at 22 000 × *g*, and then sterilised by irradiation at 40 kGy. These rumen fluids were used undiluted and without any supplementation to monitor the bacterial growth. Ammonia and lactic acid concentrations were determined according to the manufacturer's instructions (Boehringer Mannheim, Germany); pentose, hexose, and heptose concentrations as described elsewhere [3]; and concentrations of volatile fatty acids (VFA) by gas chromatography. Total ammonium content was measured using the Kjeldahl method. Titrimetry was used to reveal the buffering capacities of the rumen fluids as described by Brugère [2].

2.2. Bacterial strains and culture conditions

The characteristics of the strains are shown in Table I. All strains are phenotypically RpoS positive. NV130, NV197 and NV141 STEC strains were isolated from healthy cattle at the slaughterhouse of Clermont-Ferrand, France. CH013, CH014 and CH016 were isolated from patients suffering from HUS in the Clermont-Ferrand hospital. Bacteria were routinely cultured at 39 °C (temperature of the ruminant digestive tract) aerobically with gentle agitation (220 rpm) in a rotary incubator (Aerotron) or anaerobically: Cysteine-HCl was added (0.5 g per litre) as a reducing agent and the rumen fluid was dispensed under CO_2 into culture tubes. Since this treatment increases the acidity of the fluid, the pH was adjusted to 6 for R4 and 7 for R2 using a filter-sterilised solution of 5% bicarbonate. The pH was checked at the

Table I. STEC strains used in this study.

Strain	Serotype	Origin	stx1[a]	stx2[b]	Reference
CH013	O91:H10	HUS	N	P	[1]
NV130	O91:H10	Bovine	N	P	[10]
CH014	O91:H21	HUS	N	P	[1]
NV197	O91:H21	Bovine	N	P	[10]
CH016	OX3:H–	HUS	N	P	[1]
NV141	OX3:H–	Bovine	N	P	[10]
EDL933	O157:H7	Meat	P	P	[8]

[a] Presence of the *stx-1* gene. [b] Presence of the *stx-2* gene. P: Positive PCR. N: Negative PCR.

beginning and at the end of the experiment and it was found to be stable. STEC growth was monitored spectrophotometrically at 600 nm, using bacteria cultured overnight in Luria-Bertani (LB) medium pH 7.2, washed in saline, then diluted to OD_{600} 0.05 in LB or in undiluted pure rumen fluid. Cell survival was determined by enumeration of viable counts on a classical selective medium for enterobacteriacae, deoxycholate agar (DCA) plates. VFA concentrations were determined by gas chromatography just before inoculation of the rumen fluid and after a 24-h growth period. Sterile rumen fluid was incubated under the same conditions and used as a control.

2.3. Acid resistance assays in the abomasum fluid

Bovine abomasum fluid, pH 2.5, was collected at slaughter, gauze-filtered, centrifuged twice at 22 000 × *g* for 20 min, and then sterilised at 120 °C for 20 min. After washing in saline, overnight LB cultures were diluted to OD_{600} = 0.05 in LB, R2, or R4 medium, incubated anaerobically for 1.5 h (LB), 5 h (R2 and R4) or 24 h (LB, R2, R4). Pure abomasum fluid was inoculated with these cultures to achieve a final concentration of ca. 1×10^5 to 5×10^5 cfu/mL and incubated statically at 39 °C. Viable counts were determined immediately before acid challenge (time zero) and after incubation for 2 h at 39 °C (t_2). The cells were diluted in saline, plated on DCA plates, and incubated at 39 °C for 18 h before being counted. The percentage survival was calculated as $Nt_2 \times 100/Nt_0$, where N is the number of viable counts. The values given are averages of the results of at least three experiments.

2.4. Statistical analyses

VFA concentrations in the rumen fluids before and after STEC incubation were compared using the paired *t* test. The survival percentages of STEC in the abomasum fluid after incubation under the different experimental conditions were compared with each other. For these statistical analyses, the survival percentages were transformed into the arc sine of the square root. One-way analysis of variance was carried out by using the GLM procedure of SAS software version 6.12. Differences were considered significant for all tests if $p < 0.05$.

3. RESULTS

3.1. Characterisation of the rumen fluids

Characteristics of the rumen pools are shown in Table II. Osmolarity, NH_4 and total ammonium concentrations were very similar for each rumen pool. Lactic acid and pentoses, hexoses, or heptoses were not detected. As expected, rumen fluid collected 90 min after feeding contained a higher VFA concentration than rumen fluid collected 18 h after feeding, and rumen fluid from hay-fed sheep contained higher levels of acetate and lower levels of propionate than rumen fluid from hay/corn-fed sheep. R2 contained a higher bicarbonate concentration than R4 (not shown), probably accounting for the neutral pH of R2 compared to R4.

3.2. Growth of STEC in rumen fluid

Similar results were obtained for all seven bovine and clinical isolates tested. STEC could proliferate under aerobiosis in LB, R1, R2, and R3, but not in R4 (Fig. 1A). During growth in R1, R2, and R3, they used acetate as a carbon source (Fig. 1B). In contrast, under anaerobiosis, STEC did not proliferate but persisted in the rumen fluids up to 24 h without loss of viability as assessed by enumerating viable counts, and in these conditions, acetate was not consumed

Table II. Characteristics of the rumen pools.

Rumen-fluid pools	*R1*	*R2*	*R3*	*R4*
Diet	100% grass hay		50% grass hay/50% corn	
Collect time (hours post-feeding)	18	1.5	18	1.5
pH	7.2	6.9	7.0	6.1
Total VFA (mM)	65.1	86.5	63.1	83.6
% Acetate	76	73	62	62
% Propionate	15	18	26	28
Osmolarity ($mOsm \cdot L^{-1}$)	227	221	220	224
Ammonia (mM)	5.4	6.0	5.1	4.2
Total nitrogen ($mg \cdot g^{-1}$)	0.097	0.163	0.169	0.130
Pentoses, Hexoses, Heptoses	ND	ND	ND	ND
Lactic acid	ND	ND	ND	ND

ND: Not detected.

(data not shown). As a control, all the strains grew equally well anaerobically in the LB medium (data not shown).

3.3. Acid resistance in the abomasum

To investigate the influence of the growth medium on the acid resistance of STEC, we compared the survival of STEC cells in the abomasum content, pH 2.5, after anaerobic incubation for 5 h or 24 h in the R4 and R2 rumen fluids. As a control, cultures before challenge were also performed in the LB medium. For these experiments, we chose the reference strain EDL933 and the two O91:H10 strains, CH013 and NV130. The control experiment showed that the LB medium did not induce acid resistance to the abomasum content. However, each strain appeared to behave differently during the acid shock after incubation in R2 or R4 (Fig. 2). CH013 was equally highly acid-resistant after incubation for 5 h in R2 or R4 (44 and 31% survival, respectively), whereas NV130 and EDL933 were acid-sensitive in these conditions. After a 24 h-incubation period, the three strains were acid-sensitive after incubation in R2, whereas they presented different levels of acid-resistance after incubation in R4 (6% survival for CH013, 15% for NV130 and 33% for EDL933).

4. DISCUSSION

In the present study, our aim was to improve the understanding of the abiotic factors that influence STEC proliferation in the ruminant digestive tract by in vitro experiments. We used undiluted and unsupplemented rumen fluids collected from sheep fed different diets as the culture media for STEC. This was in contrast to other reported experiments in which rumen fluid was generally diluted in the synthetic media, and exogenous carbon sources (glucose, trypticase) were always added. The conditions used in this study, although more drastic, mimic much better the physicochemical conditions found in vivo. Our findings indicated that: (i) STEC could persist in the rumen without significant proliferation;

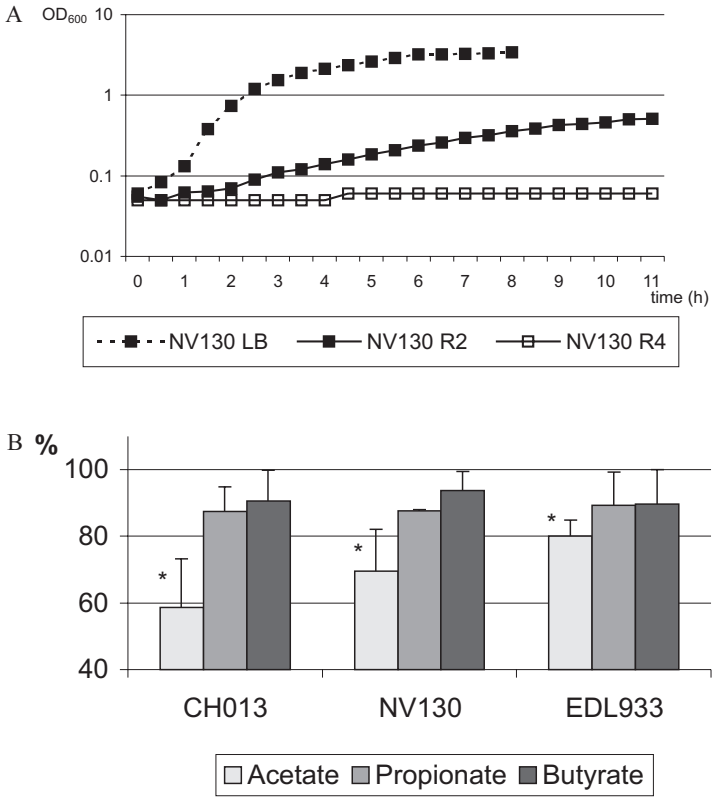

Figure 1. Growth of STEC strains in the rumen fluid under aerobiosis. A: growth curves. Only the growth curves of the O91:H21 strain NV130 in LB, R2, and R4 are shown. The growth curves of the O91:H21 strain CH013, O91:H10 strains CH014 and NV197, OX3H$^-$ strains CH016 and NV141, and O157:H7 strain EDL933 were similar. Growth curves of all strains in R1 and R3 were very similar to the growth curves in R2. B: Percentage of remaining VFAs after aerobic cultures in R2 at 39 °C of O91:H10 strains and EDL933. The results were similar for O91H21 strains. *Significantly different from 100% ($p < 0.01$) by using the Student *t* test. The results are the mean of three independent experiments.

(ii) the diet actually influenced the ruminal VFA concentrations and pH values, but acidic conditions resulting from the combination of high VFA concentration and moderately acidic pH did not alter the survival of STEC; this important finding shows that STEC strains used in this study were resistant to the effect of weak organic acids at pH 6 without the need for acid adaptation; (iii) human non-O157:H7 STEC isolates were able to persist in the rumen contents in spite of low oxygen availability and nutrient deprivation, in the same manner as bovine STEC isolates did. On the whole, these results indicate the potential role of the rumen as a reservoir for STEC strains which are pathogenic for humans. Comparison of STEC growth under aerobiosis and anaerobiosis suggests that at least two different factors could limit STEC proliferation in the rumen: nutrient limitation, since anaerobiosis prevents the utilisation

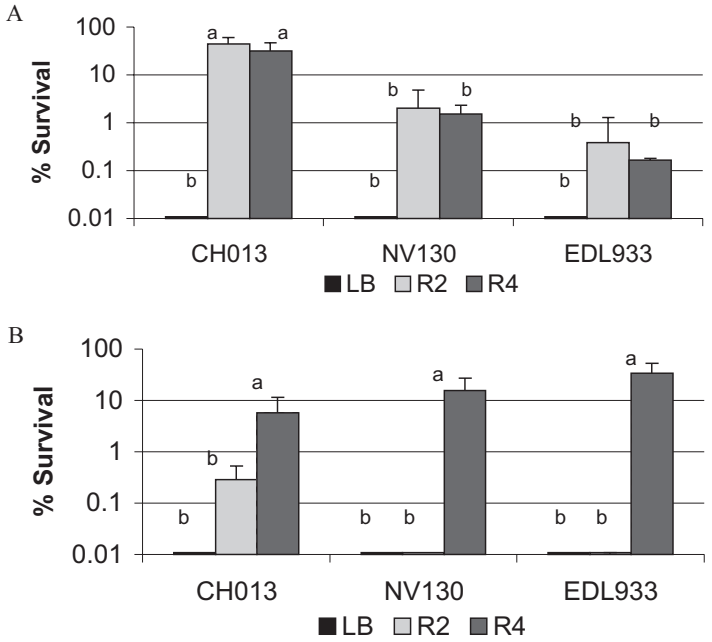

Figure 2. Resistance of STEC cells to the acidic conditions of the abomasum. A: Cells were incubated in anaerobiosis for 1.5 h in LB, 5 h in R2 or R4. B: Cells were incubated for 24 h in anaerobiosis in LB, R2 or R4. The acid challenge was performed by incubating 1×10^5 to 5×10^5 cells for 2 h in abomasum fluid pH 2.5. The differences were considered significant if p values < 0.05. a is significantly different from b. Results are the mean of at least three independent experiments.

of acetate as a carbon source, and acid stress due to corn feeding, resulting from the combination of high VFA concentration with moderately low pH. Future experiments should focus on the interactions between STEC and the commensal flora which could also have significant effects on STEC survival in the rumen compartment [4, 14].

STEC cells should cross the highly acidic barrier of the abomasum (pH 2.5) before reaching the gut. We thus investigated whether cells incubated anaerobically for 5 h or 24 h in the rumen fluid are resistant to the abomasum juice. Since STEC did not proliferate under these conditions, these incubation times did not correspond to a particular phase of growth, but to the minimal and maximal duration that the STEC might reside in the rumen before reaching the abomasum. Our results showed that STEC resistance to the acid stress encountered in the abomasum fluid was not constitutive (in contrast to the resistance to the acid stress due to VFA and acidic pH) but must be induced by environmental factors not present in the LB medium. The medium that STEC experience before suffering the acid stress in the abomasum influences their survival: 24 hours in the R4 medium induced a higher level of acid resistance. The acid stress encountered in R4, due to the high concentration of VFA combined with pH 6, was likely to account for the induction of this mechanism of acid adaptation. STEC cells that have been acid-adapted in the rumen of grain-fed cattle will probably better survive

carcass decontamination, food processing, and the gastric acidity barrier of humans, and therefore will present a substantial risk to human health. However, much more information about in vivo induction and maintenance of STEC acid resistance, and about the in vivo physiology and ecology of STEC cells in the rumen and the colon, is needed before recommending a diet.

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