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Role of Competition for Substrate in Bacterial Antagonism in the Gut

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Small slices of agar, each containing about $10^{5.5}$ *Escherichia coli* organisms, were preincubated either in the contents of rat cecum or in brain heart infusion broth for 2 h and then were transferred to a small sample of saline. The purpose of these experiments was to examine the ability of *E. coli* to grow on the substrate that penetrated from the contents of rat cecum into the agar slices. It appeared that preincubation in brain heart infusion broth gave rise to abundant growth, whereas only poor growth occurred after preincubation in the contents of rat cecum. This poor growth was completely reversed by adding brain heart infusion. The same experiments were repeated under anaerobic conditions, in which growth on substrate that penetrated from the contents of rat cecum into the agar slices was extremely poor. This extremely poor growth under anaerobic conditions was reversed to abundant growth by adding brain heart infusion broth. The addition of nitrate as an electron acceptor also stimulated growth of *E. coli*. Similar results were obtained with other bacteria and with human feces. The results can be interpreted as a demonstration that under anaerobic conditions such as occur in large intestines, bacterial antagonism is caused in a high degree by competition for substrate.

The selective inhibition of growth of aerobic and facultative anaerobic bacteria, called colonization resistance (11), in the large intestines of mammalian species including humans has been discussed for many years. It has often been suggested that volatile fatty acids (VFA) are important growth-inhibiting factors in the gut, in combination with low pH values and low oxidation-reduction potential (1, 2, 9). However, it has been shown in our laboratory that VFA are not important in this respect because the number of aerobic bacteria in the rat gut is not correlated with the pH, the VFA concentration, or the oxidation-reduction potential in the cecum (H. F. L. Guiot and R. van Furth, submitted for publication). Furthermore, during a 6-h period after the injection of about 10^6 *Escherichia coli* organisms into the cecal cavity of live rats, the number of these bacteria did not increase. The mean growth rate during this incubation period was nil and was unaffected by substantial changes in the concentration of undissociated VFA molecules, which are the active inhibitory components in vitro (Guiot and van Furth, submitted for publication). These findings suggest that other factors are involved in the selective inhibition of growth, i.e., factors which have a stronger inhibitory activity and are therefore capable of masking the postulated effect of VFA. No indications were found that a toxic compound was responsible for masking the postulated VFA effect (Guiot and van Furth, submitted for publication).

One of the alternative hypotheses put forward to explain the selective inhibition of growth of aerobic bacteria and facultative anaerobes in the gut is bacterial antagonism owing to competition for substrate. Our earlier findings (Guiot and van Furth, submitted for publication) indicate that the nutritional reserve for bacteria in the contents of the rat cecum is probably small. For instance, one night of fasting led to a substantial decrease in the concentration of VFA, which probably reflected a decrease in the bacterial nutritional reserve owing to a decreased supply of food and to secretions from the small intestine into the cecum.

The experiments reported here investigated whether nutritional conditions in the cecal contents of rats and in human feces can explain the selective inhibition of aerobic bacteria and facultative anaerobes.

MATERIALS AND METHODS

General design. An 18-h culture of *E. coli* of serological type 0.11 K^-, insensitive to kanamycin and cefaloridine (i.e., no inhibition zone for 30-μg disks of the two drugs), was grown in brain heart infusion (BHI)

broth (Oxoid Ltd., London, England) and diluted in saline (1:10), and 0.1 ml of the diluted suspension was added to 10 ml of melted (45°C) 1% agar–saline. The bacterial suspension was then homogenized in a Vortex mixer and was poured into a glass tube (inner diameter, 10 mm). After the agar solidified, it was driven out of the tube by blowing, and the agar cylinder was cut into slices about 4 mm thick, yielding disks of about 0.4 g each. A total of 14 such slices, each containing about $10^{5.5}$ *E. coli* organisms, were preincubated together for 2 h at 37°C in either 10 ml of BHI broth or 10 ml of diluted cecal contents of rats (8 g of cecal contents, 2 ml of saline) in diluted human feces (8 g of feces, 2 ml of saline) or in the dialyzable fraction of human feces. Next, the agar slices were removed from the preincubation medium, washed in saline, submerged individually in either 1 ml of saline or 1 ml of BHI broth, and incubated at 37°C. At 2-h intervals, slices were removed from the incubation medium to determine the number of *E. coli* colony-forming units (CFU) per agar slice, either in selective solid BHI (containing 30 μg each of kanamycin and cefaloridine per ml) or in nonselective solid BHI. Similar experiments were performed with *Klebsiella pneumoniae* and *Streptococcus faecalis*.

Experiments with cecal contents. Cecal contents were obtained from conventional white rats (Cpb-Wu, Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands), from white rats after a 20-h fast, or from rats pretreated with antibiotics. The rats were killed by ether inhalation, and the cecal contents were removed for preparation as preincubation medium. The rats to be pretreated with antibiotics were anesthetized by ether inhalation, and the abdomen was opened by incision along the linea alba, after which 0.5 ml of antibiotic solution (300 μg each of kanamycin and cefaloridine per ml) was carefully injected into the duodenal cavity and 1 ml was injected into the cecal cavity. To permit the effects of the surgical intervention to be recognized, rats injected with saline alone were used as controls.

After the injection, the abdomen was closed with Ethicon Leinenzwirn 60 (Ethicon GmbH, 2000 Norderstedt, West Germany), and the rats were allowed to recover consciousness. After 18 h, they were killed by ether inhalation, and the cecal contents were removed to be used as preincubation medium. In all experiments, the pH of the cecal contents was adjusted to 7.5 (in NaOH) before the agar slices were added.

Parts of the experiments were performed under anaerobic conditions, with the air in the test tubes removed by the inflow of N_2 gas (10% CO_2) and the tubes containing the incubation medium heated before use (60°C). In one of these anaerobic experiments, nitrate was added to the saline as an electron acceptor (0.5 mg of $NaNO_3$ per ml).

Experiments with human feces. Similar experiments were performed with human feces as the preincubation medium. The source of feces was a healthy subject, and the pH was adjusted to 7.5 (with 1 N NaOH) before the agar slices were added. A similar experiment was performed with the dialyzable fraction of the extract of feces from the same subject. For this purpose, after the addition of 10 ml of demineralized water, 30 g of feces (wet weight) was homogenized (shaken by hand for 1 min), the mixture was centrifuged for 15 min (4.9 × *g* at room temperature), and the supernatant was dialyzed (Visking 2 Å, Höfelt, The Hague, The Netherlands) at 2°C for 18 h against an equal volume of demineralized water. This dialyzable fraction was used for preincubation under aerobic and anaerobic conditions. In the latter case, air was removed by the inflow of N_2 gas (10% CO_2) from the tubes containing the preincubation medium as well as from the incubation medium.

Viable counts. The agar slices were removed from the preincubation or incubation media, washed in saline, and homogenized with a Potter homogenizer that contained a final volume of 5 ml of demineralized water. The homogenized suspension was diluted by 10-fold steps in saline, and the number of CFU was determined in solid BHI (1% agar no. 1, Oxoid Ltd.).

Characterization of growth curves. The results of the various experiments were evaluated by comparing curves of the number of CFU plotted against the corresponding time intervals. These growth curves are characterized by a lag phase, a mean maximal growth rate constant (K_{max}), and a yield (Y). The lag phase was defined as the time needed (preincubation included) for the bacterial strain in question to start growing at K_{max}. The K_{max} was calculated from the part of the growth curve with the greatest inclination; at that part a tangent line was constructed, and from the inclination K_{max} was calculated with the equation $K = (\log N_{t2} - \log N_{t1})/(\log 2t)$ where K is the exponential growth rate constant, N_t is the CFU count at time t, and t is the corresponding time interval in hours. Y was defined as the CFU count in agar slices incubated for 20 h (2 h of preincubation included).

RESULTS

Growth of *E. coli* in medium containing BHI. To obtain some information about the amount and diffusion rate of nutrients into the agar slices, the growth of *E. coli* in slices consisting of solid BHI was first compared with growth in saline-agar slices preincubated in BHI broth for 2 h. *E. coli* embedded in solid BHI started after a 1-h lag phase to grow at a mean K_{max} of 2.3 generations per h, resulting in a mean Y of $10^{9.2}$ CFU per agar slice (Table 1, Fig. 1). In the saline-agar preincubated in BHI broth, growth occurred after a 3-h lag phase at a mean K_{max} of 2.3 generations per h, resulting in a mean Y of $10^{8.4}$ CFU per agar slice (Table 1, Fig. 1).

Growth of *E. coli* on substrate derived from cecal contents of rats. In the next experiment, *E. coli* was embedded in saline-agar slices that were preincubated in the cecal contents of rats to examine whether nutrients were present in the cecal contents. To find out whether the slices were contaminated with bacteria from the cecal contents, CFU counts were performed both in solid BHI and in selective solid BHI containing kanamycin and cefaloridine. No differences were found between the results in selective and nonselective BHI.

The agar slices were incubated in either saline or BHI broth. In saline, growth was characterized by an 8-h lag phase, a mean K_{max} of 1.2

TABLE 1. Growth characteristics of *E. coli* embedded in agar slices[a]

Medium		Growth		
Preincubation	Incubation	Lag phase (h)[b]	K_{max} (generations per h)	Y (CFU per slice)
None	Saline	1	2.3	$10^{9.2}$
BHI broth	Saline	3	2.3	$10^{8.4}$
Cecal contents of rats	BHI broth	5	2.3	$10^{9.0}$
Cecal contents of rats	Saline	8	1.2	$10^{7.3}$
Cecal contents of antibiotic-treated rats	Saline	6	1.7	$10^{8.0}$
Cecal contents of rats	Anaerobic saline	8	0.7	$10^{6.4}$
Cecal contents of rats	Anaerobic saline–$NaNO_3$	8	1.4	$10^{7.6}$
Cecal contents of rats	Anaerobic BHI broth	8	2.0	$10^{8.8}$
Human feces	BHI broth	4.5	2.3	$10^{9.0}$
Human feces	Saline	7	1.2	$10^{7.3}$

[a] Agar slices of 0.4 g contained about $10^{5.5}$ CFU of *E. coli*. Except in the experiment without preincubation, in which the agar slices contained BHI, all slices were made of saline.
[b] Includes preincubation time.

generations per h, and a mean Y of $10^{7.3}$ CFU per agar slice (Table 1, Fig. 2). Similar values were obtained with slices preincubated in the cecal contents of rats that had fasted overnight before the experiments (Fig. 2). The addition of substrate during the incubation period by substituting BHI broth for saline improved growth; the lag phase was reduced from 8 to 5 h, the mean K_{max} increased from 1.2 to 2.3 generations per h, and the mean Y rose from $10^{7.3}$ to $10^{9.0}$ CFU per agar slice (Table 1, Fig. 2).

Effect of antibiotic treatment of rats on the availability of bacterial substrate. The experiments were repeated with the cecal contents of rats that had been injected with either an antibiotic solution or saline in the lumen of the gut. The results for *E. coli* preincubated in the cecal contents of rats injected with saline were similar to the corresponding results with untreated rats (compare Fig. 2 and Fig. 3). Antibiotic treatment improved conditions for growth; growth occurred after a 6-h lag phase with a K_{max} of 1.7 generations per h, resulting in a Y of $10^{8.0}$ CFU per agar slice (Table 1, Fig. 3).

Effect of anaerobic incubation on growth. A part of the experiments described above was repeated under anaerobic conditions with or without the addition of nitrate to the saline as an electron acceptor. Anaerobic incubation of agar slices in saline after preincubation in the contents of rat cecum resulted in very poor growth: an 8-h lag phase, a K_{max} of 0.7 generations per h, and a Y of $10^{6.4}$ CFU per agar slice (Table 1, Fig. 4). Growth characteristics were similar when the agar slice contained $10^{4.5}$ instead of $10^{5.5}$ CFU per agar slice. The remarkable difference was that growth continued longer, until the same Y was reached as for agar slices which contained $10^{5.5}$ CFU per agar slice. Adding $NaNO_3$ to the saline improved the conditions for growth of *E. coli* with a K_{max} of 1.4 generations per h and a Y of $10^{7.6}$ CFU per agar slice. Adding substrate during the incubation period by substituting BHI broth for saline substantially improved the conditions for growth, with a K_{max} of 2.0 generations per h and a Y of $10^{8.8}$ CFU per agar slice (Table 1, Fig. 4).

Growth on substrate derived from human feces. The effect of preincubating agar slices with *E. coli* in human feces was approximately the same as that of preincubating them in the cecal contents of rats. When the slices were added to saline, growth occurred after a 7-h lag phase, with a mean K_{max} of 1.2 generations per h and a mean Y of $10^{7.3}$ CFU per agar slice (Table 1). Growth again improved when the agar slices were added to BHI broth instead of to saline; the lag phase was 4.5 h, the K_{max} was 2.3 genera-

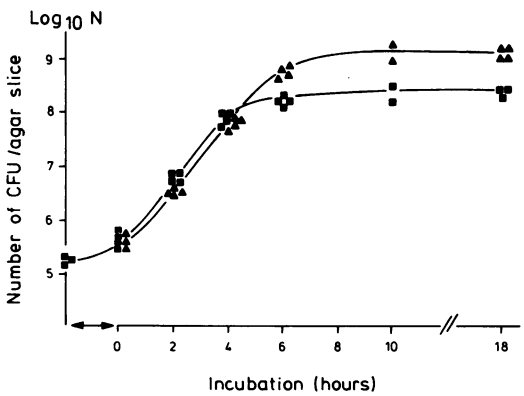

FIG. 1. Growth of *E. coli* in saline-agar slices preincubated in BHI broth and incubated in saline (■), and in BHI-agar slices not preincubated but directly incubated in saline (▲). Double arrow in this and subsequent figures indicates preincubation period (2 h).

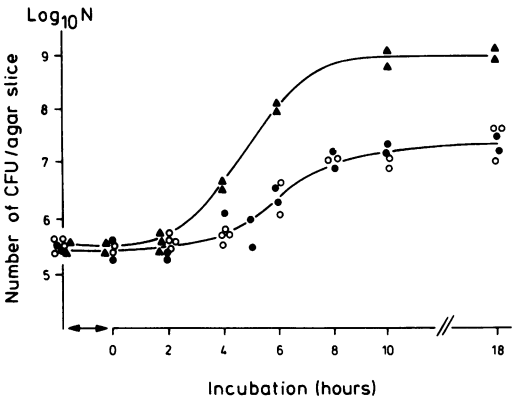

FIG. 2. Growth of *E. coli* in saline-agar slices preincubated in the cecal contents of rats and incubated in either BHI broth (▲, untreated rats) or in saline (●, untreated rats; ○, unfed rats).

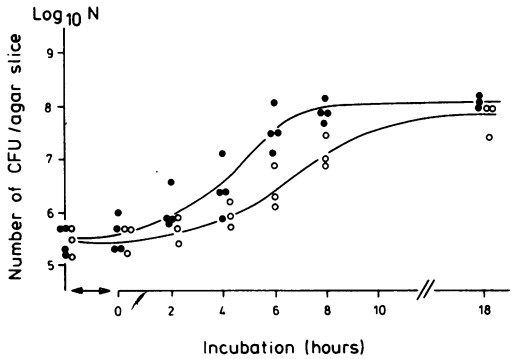

FIG. 3. Growth of *E. coli* in saline-agar slices preincubated in cecal contents of rats pretreated with saline (○) or antibiotics (●). Slices were next incubated in saline.

tions per h, and the Y was 10^9 CFU per agar slice (Table 1).

Preincubation of agar slices containing *Klebsiella pneumoniae* or *S. faecalis* in human feces had a similarly unfavorable effect that could be reversed by incubation in BHI broth, although the individual growth characteristics differed from those obtained with *E. coli* (Fig. 5).

Effect of anaerobic conditions on growth of *E. coli* on human fecal substrate. It was expected that growth on substrate composed mainly of high-molecular-weight compounds would be more impaired by anaerobic conditions than it would be with low-molecular-weight substrate. Therefore, an investigation was made to determine whether growth would also be impaired by anaerobic conditions in an experiment with the dialyzable (low-molecular-weight) fraction of human feces. It is remarkable that preincubation in the dialyzable fraction resulted in a relatively short lag phase of 3 h, a mean K_{max} of 1.6 generations per h, and a mean Y of $10^{8.0}$ CFU per agar slice (Fig. 6). A decrease in the mean Y from $10^{8.0}$ to $10^{7.1}$ CFU per agar slice was the main result of incubation under anaerobic conditions (Fig. 6).

DISCUSSION

In the last few years, selective gut sterilization seems to have become more generally accepted as a method for preventing infection in patients with decreased host resistance (3, 5–8, 10, 12). Therefore, attention has to be focused on bacterial antagonism in the gut because this phenomenon is an important starting point for this method of preventing infection. If potentially pathogenic aerobic bacteria are eliminated selectively from the gut, fewer infections will occur since bacterial antagonism helps to prevent recolonization by these potential pathogens (9, 11). It has been suggested that the products of anaerobic metabolism, i.e., VFA and low pH, are the main factors responsible for the selective inhibition of growth of aerobic or facultative anaerobic bacteria (1, 2, 9). Previous results obtained in our laboratory (Guiot and van Furth, submitted for publication) did not support this suggestion. The cause of the selective inhibition of growth was therefore sought in other factors with stronger inhibitory effects which probably masked the postulated effect of the pH and VFA.

One possible explanation of bacterial antagonism in the gut is bacterial competition for substrate. Competition has already been suggested as a mechanism for bacterial antagonism by Freter (4), and the kinetics of intestinal

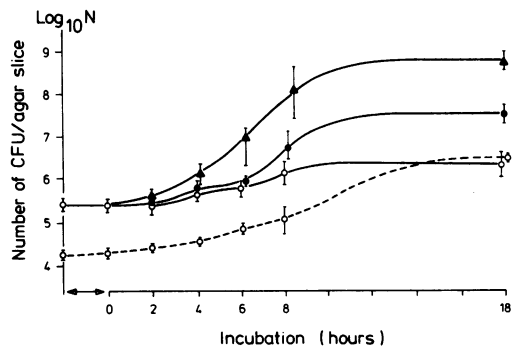

FIG. 4. Growth of *E. coli* in saline-agar slices preincubated in the cecal contents of rats and incubated under anaerobic conditions. Symbols: ○, incubated in anaerobic saline (mean and range of four experiments; dashed line shows results with slices containing $10^{4.5}$ CFU); ▲, incubated in anaerobic BHI broth (mean and range of four experiments); ●, incubated in anaerobic saline with $NaNO_3$ (mean and range of five experiments).

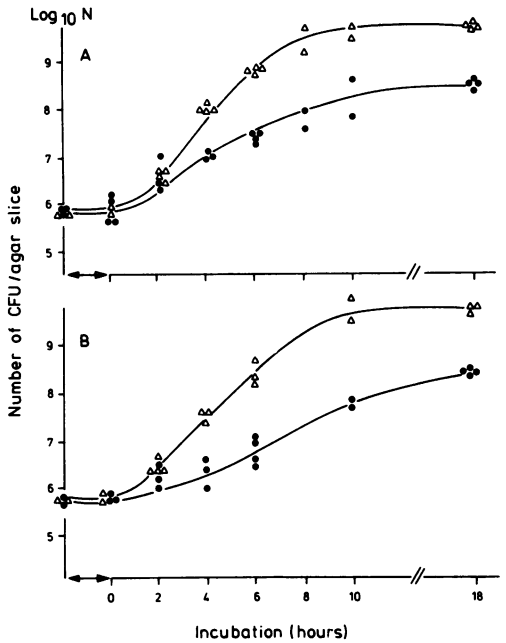

FIG. 5. Growth of *K. pneumoniae* (A) and *S. faecalis* (B) in saline-agar slices preincubated in human feces and incubated in either BHI broth (△) or saline (●).

bacteria make it, theoretically, an important growth-controlling mechanism (Guiot, submitted for publication). The complex anaerobic population of the gut, in which bacteria of several species are present in high numbers, would use an appreciable part of the bacterial substrate present in the contents of the large intestines.

The experimental methods used were chosen with the idea that agar slices would be the best system for giving information about the presence of growth-limiting toxic compounds and about the quantity of nutrients present in the fresh untreated contents of rat cecum. Contamination of agar slices with bacteria from the cecal contents was probably minimal because no differences were observed between counts performed in selective and nonselective solid BHI.

The results of the present study indicate that nutritional conditions in the cecal contents of rats are not optimal for the growth of *E. coli* and that conditions in human feces are not optimal for the growth of *E. coli*, *K. pneumoniae*, and *S. faecalis*, as shown by the experiments in which the contents of rat ceca and human feces were used as preincubation media. When the bacteria in question were transferred from the preincubation medium into saline, growth was relatively poor, whereas transfer to BHI broth instead of saline produced a growth pattern with a shorter lag phase, a higher K_{max}; and a Y (Table 1). If the relatively poor growth during incubation in saline had been caused by toxic compounds penetrating from either substrate into the agar slices during preincubation, incubation in BHI should have led to relatively poor growth also. In contrast, under the latter conditions the growth characteristics of *E. coli* were similar to those found in unpreincubated BHI agar slices, except for the lag phase (Table 1). However, the 5-h lag phase (preincubation included) shown by *E. coli* preincubated in cecal contents and incubated in BHI can be explained as the time needed for the nutrients to penetrate into the agar slices in adequate concentrations. Measured from the first contact with BHI broth, at time zero the lag phase for *E. coli* was about 3 h (preincubation excluded), which is similar to the 3-h lag phase for *E. coli* preincubated in BHI broth and incubated in saline (Fig. 1).

Although the results discussed so far suggest that nutritional conditions in the cecal contents are not optimal, a considerable increase from $10^{5.5}$ to $10^{7.3}$ CFU per agar slice still occurred. This is not in accordance with the results of previous studies in our laboratory (Guiot and van Furth, submitted for publication), indicating that the *E. coli* strain which we used was not at all able to grow in the ceca of conventional rats. In addition, the possibility of toxic compounds is not ruled out because these postulated compounds might have been active in the slices incubated in saline but inactivated by compounds in BHI broth. The experiments performed under anaerobic conditions present a more definite solution. When the agar slices were transferred from the cecal contents to anaerobic saline, the growth of *E. coli* decreased in comparison with the corresponding results under aerobic conditions (compare Fig. 2 and Fig. 4). The K_{max} decreased from 1.2 to 0.7

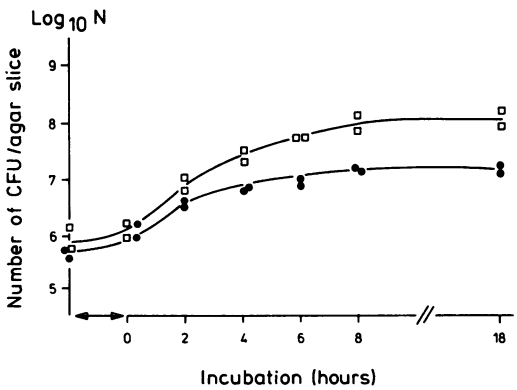

FIG. 6. Growth of *E. coli* in saline-agar slices preincubated in the dialyzable fraction of human feces and incubated in saline under aerobic (□) or anaerobic (●) conditions.

generations per h, and Y decreased from $10^{7.3}$ to $10^{6.4}$ CFU per agar slice simply because oxygen was removed from the saline. With anaerobic BHI as the incubation medium, growth became abundant; although the lag phase was much longer, the K_{max} and the Y were about the same under aerobic and anaerobic conditions.

The observations indicate that the nutritional conditions in the cecal contents are poor, but they become extremely poor in the absence of oxygen. With the addition of nitrate as an alternative electron acceptor instead of oxygen, growth conditions improve. This observation precludes explaining the results by means of toxic compounds. It is hard to imagine why these postulated compounds should be less active in the presence of BHI and $NaNO_3$ alike.

Actually, the results with agar slices containing $10^{4.5}$ instead of $10^{5.5}$ CFU apiece rule out the involvement of a toxic compound. If a toxic compound was involved, a complete growth inhibition should have been observed after 8 h of incubation, similar to the results with the agar slices that contained $10^{5.5}$ CFU of *E. coli* apiece. In contrast, growth continued until the same Y of about $10^{6.4}$ CFU per agar slice was reached, indicating that the nutritional conditions became growth limiting at that level.

The experiments with rats treated with kanamycin and cefaloridine were performed to assess the effects of treatment with antimicrobial agents on the bacterial substrate conditions for *E. coli* in the cecum. Direct administration of the antibiotics by injection was preferred to oral administration because of standardization and the need for a valid control group, realized by the replacement of antibiotic solution with saline. The results show that the antibiotic treatment led to an improvement of the bacterial substrate conditions; *E. coli* grew better on substrate derived from the ceca of rats treated with antibiotics than on substrate derived from the ceca of untreated rats or of rats treated with saline.

The results of the experiments that were described in the present paper indicate that the combination of poor nutritional conditions and the lack of electron acceptors are probably important growth-controlling factors in the intestines of both rats and humans.

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