Interactions of *Vibrio cholerae, Shigella flexneri,* Enterococci, and Lactobacilli in Continuously Fed Cultures. (26617)

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The factor or factors precipitating signs and symptoms of clinical cholera in man are not well-defined. Attributes of the organism itself have been repeatedly invoked in the 76 years since Koch's isolation of the vibrio. Lankford(1) has recently reviewed the significant findings in this field. Similarly, Pollitzer(2,3) has reviewed the literature with respect to characteristics of the host which may be of significance in susceptibility of man to infection with *Vibrio cholerae.*

The possibility has been considered that the normal flora of the intestinal tract might be significant in susceptibility to infection with enteric pathogens. Freter(4,5) has demonstrated that infections with *Shigella flexneri* and *V. cholerae* could be established in the intestines of normally resistant mice and guinea pigs when the intestinal microflora were reduced or eliminated by previous treatment with antibiotics, and that such infections could be eliminated by the simultaneous administration of viable cells of *Escherichia coli*(5,6). However, cholera is an infection of the small intestine and Lankford(1) has pointed out that the absence of *E. coli* from this region in man and laboratory mammals would cast doubt upon its presumed ability to prevent infection by direct antagonism.

Unlike *E. coli,* lactobacilli are common inhabitants of the upper as well as the lower intestinal tract of laboratory animals(9,10, 11). The same is also true of Group D streptococci or enterococci, though perhaps not universally present or present in lesser numbers(9,10,11). In addition, lactobacilli have been reported to influence the course of infection(7) or to be capable of producing antibiotics(8). We therefore selected these 2 types of organisms for a study of interactions *in vitro.*

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The technic of continuous culture chosen to carry out this study was used by Freter (6,11) to evaluate interactions between strains of coliform bacteria and enteric pathogens. He found inhibition of shigellae and cholera vibrios by this means when other methods failed. These findings emphasize the importance of studying bacterial interactions in an environment dynamically similar to the native ecological niche. This is particularly true with regard to enteric pathogens which multiply and cause their effects in the lumen of the gut, where conditions are constantly changing; new nutritional substances continually are coming in, while spent materials and parts of the bacterial population are departing. In this paper certain interactions of indigenous biota of the intestine and 2 enteric pathogens, *Vibrio cholerae* and *Shigella flexneri* 2a are examined.

Methods and materials. The apparatus designed for continuous culture(17) embodies a number of principles set forth by others(12, 13, 14, 15, 16). The general subject has been reviewed by Novick(16). A diagram of the apparatus used in this work is given in Fig. 1.

The experiments were conducted by setting up pairs of growth tubes, each receiving an identical flow of fresh medium, and draining through rubber tubing into a common waste receptacle. Under aerobic operations, the glass tube (K, Fig. 1) was plugged with cotton and used as a sampling port. When anaerobic operation was desired, this tube was closed with a vaccine vial stopper. A flow of nitrogen was then introduced through a heavy gauge (18 or 19 ga.) hypodermic needle inserted into the vaccine vial stopper. A steady flow of gas (5% CO_2, 95% N_2) had no deleterious effect on the operation of the system.

Flow rates were adjusted to 74-76 ml/hr.

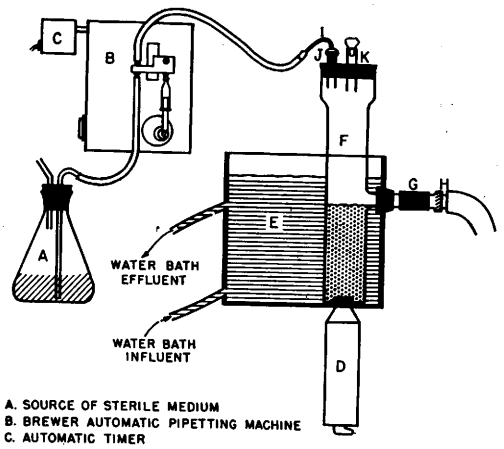

FIG. 1. Diagrammatic sketch of continuous culture apparatus.

The effective capacity of the growth tubes used was 79-81 ml. Thus, the turnover of medium in the growth tubes was approximately 100% per hour. Under these conditions, samples were taken by puncturing, with aseptic technic, the rubber tubing connected to the side-arm, using a sterile hypodermic needle and syringe.

Cultures: Vibrio cholerae (Inaba) strain 20-A-67 was obtained from the WRAIR collection. *Shigella flexneri* 2a strain 2457 was isolated in 1954 by Lt. Colonel Oscar Felsenfeld from a dysentery outbreak in Japan. They were maintained on meat extract agar kept at 5°C. Transfers of the stock cultures were made at weekly intervals. Inocula for continuous culture experiments consisted of 1 ml of a dilution of agar grown suspension of organisms containing 10^2 to 10^3 viable cells.

The enterococcus culture employed was a Group D streptococcus isolated from a human source. The culture was provided by Dr. James Rust, Dept. of Bacteriology, WRAIR.

The culture designated as "lactobacillus" was isolated from a normal guinea pig small intestine. This organism grows readily on a selective medium(18), is peroxidase-negative and cytochrome-negative as tested by the method of Deibel and Evans(19). The organism is a Gram-positive, non-spore-forming, non-motile rod. It grows poorly under strictly aerobic conditions. The criteria mentioned above are considered sufficient to identify this organism as a lactobacillus type.

Growth medium: The medium used for growth in continuous culture was essentially Bacto-Thioglycollate Medium. The agar was omitted from this medium and glucose was added to a concentration of 0.1%. In addition, the medium was buffered with phosphate buffer to a pH of 6.9-7.2 after autoclaving. This step was found necessary to maintain the pH at suitable levels.

Recovery media: For plate counts of *V. cholerae* and *Sh. flexneri* 2a, a meat extract agar of the following composition was used:

Beef extract	3 g	NaCl	5 g
Bacto-peptone	10 g	2N NaOH	3 ml
Bacto-agar	20 g	Distilled water	1000 ml

For the enterococcus, Brain Heart Infusion Agar (BHIA) (Difco) was used.

For the lactobacillus, the Lactobacillus selection medium of Baltimore Biological Laboratories, Inc. was employed.

All plate counts were performed by the method of Miles *et al.*(20). The plate counts for *V. cholerae* were incubated 18-24 hours aerobically at 37°C. Under these conditions, the lactobacilli did not grow at all within this time period, and the enterococci, although forming a slight background of growth did not interfere with enumeration of the cholera colonies. Preliminary experiments indicated that *V. cholerae* or *S. flexneri* counts from mixed cultures were equivalent to those obtained in pure culture. The coefficient of variation of the method ranged from 3 to 30%.

More difficulty was encountered in enumerating enterococcus colonies on BHIA. On this medium the vibrio or shigella colonies grew as well as the enterococci; however the latter were readily distinguished on the basis of colony morphology. Preliminary studies using a selective medium for the enterococci (SF medium, Difco) indicated that counts of the enterococcus on BHIA in presence of vibrio or shigellae were equivalent to those obtained on the selective medium. The latter

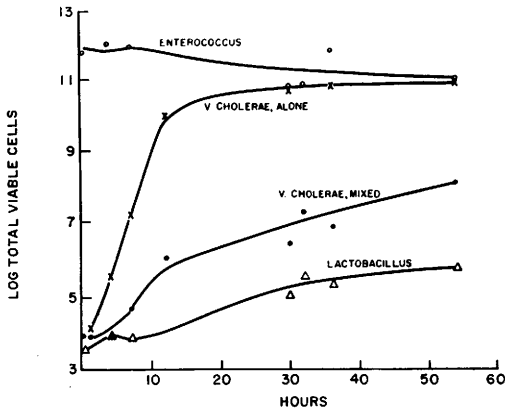

FIG. 2. Effect of enterococcus and lactobacillus growth of *Vibrio cholerae* in continuous culture.

was not employed because of the long incubation period (48-72 hours) necessary to obtain a count.

Plate counts of lactobacilli made on LBS medium were incubated 48 hours at 37°C in candle jars. Under these conditions. the other organisms produced no visible growth.

Static Stirred Cultures: For certain experiments, pure and mixed cultures were grown in 25 × 200 mm test tubes. Magnetic stirring bars were always used to maintain homogeneity of the cultures. Conveniently, these tubes could be placed in the water bath of the continuous culture apparatus over the magnetic stirring motor.

Chick Embryo Studies: Experiments with *V.* cholerae and the enterococcus culture were carried out in the chick embryo employing procedures already described(21).

Results: In the first experiment, the enterococcus and the lactobacillus strains were grown together in one tube of the continuous culture apparatus until each reached a steady state. At this time, equal numbers (10^2-10^3) of *V. cholerae* were added to the mixed culture and also to an uninoculated tube. The numbers of organisms in each tube were determined over a 54 hour period. Results are summarized in Fig. 2. Throughout the 54 hour period of observation, the numbers of viable cells of enterococci remained high and almost constant (*ca.* 10^{12} cells total). The lactobacilli, however, remained at low levels (10^4-10^5), but increased slightly between 36 and 54 hours.

The *V. cholerae* cells in pure culture went into a phase of exponential growth after a short lag period of approximately one hour, and reached a steady state after 12 hours at 10^{10}-10^{11} cells. One must bear in mind in interpreting Fig. 2, that the cells were not only increasing in numbers or remaining constant, but were keeping up with the constant influx of fresh medium and loss of some cells, amounting to nearly a complete turnover in volume of the growth tube each hour. Table I shows the calculated generation times required for each organism to achieve the performance shown in Fig. 2.

In the mixed culture, the *V. cholerae* growth exhibited a longer delay, followed by a gradual increase to a level of 10^6-10^7 cells, a level some 10,000-fold lower than that achieved by sister cells in pure culture.

Although the culture containing the mixed growth of enterococci and lactobacilli maintained a reasonably constant pH of 6.5-6.6 during the preliminary period of incubation, it showed some fluctuation after addition of the vibrios, and was not always identical with the pure vibrio culture. Therefore, an experiment was conducted in static stirred cultures to determine the effect of various pH levels on growth of the vibrio. Aliquots of medium were adjusted to pH 7.00, pH 6.32, and pH 6.12 with phosphate buffer and seeded with

TABLE I. Generation Times of *Vibrio cholerae* in Continuous Culture.

Time (hr)*	Generation times (min.)† Pure	Mixed with enterococcus
0 - 1	57.7	52.6
1 - 3½	16.0	43.4
3½ - 7	14.3	29.5
7 -12	17.3	24.2
12 -30	44.7	42.0
30 -36½	43.6	42.0
36½-54	43.6	35.6

* After inoculation of *V. cholerae.*

† Time required for population to double, including correction for flow rate, *i.e.*

$$\text{Doubling time} = \ln 2 \Big/ \frac{\ln N_2 - \ln N_1}{t_2 - t} + \frac{w}{v},$$

where N_2 = population at time t_2
N_1 = population at time t
w = flow rate
v = vol of growth tube

TABLE II. Effect of Variation of pH on Growth of *Vibrio cholerae* in Static Culture.

Time after inoc. (hr)	Viable cells/ml Starting pH of medium 7.00	6.32	6.12
0	1.00×10^3	1.00×10^3	1.00×10^3
2	4.45×10^2	4.45×10^2	1.78×10^2
6	5.30×10^6	1.47×10^6	3.26×10^5
13	6.82×10^8	6.45×10^8	6.25×10^8
28	4.90×10^8	5.58×10^8	7.35×10^8

identical inocula. The results are shown in Table II. The slight differences observed were not sufficient to account for the results obtained in continuous culture.

A second experiment was carried out in continuous culture to determine if enterococci alone could inhibit growth of *V. cholerae* to the degree achieved by the combination of lactobacillus and enterococcus. The results were similar to the experiment summarized in Fig. 2. When the experiment was carried out under anaerobic conditions, inhibition of *V. cholerae* was also observed. Under aerobic conditions, maximum cell yield of the pure culture of cholera was 8.72×10^8 while under anaerobic conditions, maximum cell yield was 1.91×10^9. This difference in the pure cultures is not considered significant. However, maximum cell yield of the *V. cholerae* in mixed culture was 2.77×10^6 under aerobic conditions and 8.55×10^4 cells under anaerobic conditions.

Two experiments similar to those described above were conducted on mixed cultures of enterococci and *Shigella flexneri* 2a, one under aerobic and one under anaerobic conditions. In neither case was a significant difference in the control shigella culture and the combined enterococcus-shigella culture demonstrable.

The ability of the enterococcus to protect embryonated eggs against an experimental cholera infection was tested by technics previously described(21). Twelve-day-old chick embryos were inoculated allantoically with approximately 10^6 enterococcus cells and groups were challenged 24 hours later with graded doses of *V. cholerae*. The data are summarized in Table III. Control eggs inoculated only with dilutions of *V. cholerae* succumbed rapidly; the LD_{50} at 48 hours approximated 3.5 organisms. The enterococcus is not pathogenic for chick embryos and did not kill any eggs. However, previous infection with enterococci conferred a degree of resistance against subsequent challenge with *V. cholerae*. Other strains of group D streptococci exhibit this effect to varying degrees. In a later experiment, no protection was obtained when graded doses of cholera vibrios were inoculated immediately following the enterococci. Thus the results are similar to those of Finkelstein and Ransom (21) with *E. coli* in which it was demonstrated that endotoxin was the resistance promoting factor. To our knowledge there have been no reports of endotoxin-like factors in fecal streptococci. Lactobacilli (9 strains) apparently do not multiply in embryonated eggs and do not protect against *V. cholerae* even when heavy doses were administered 24 hours prior to challenge.

Discussion. A true steady state of exponential growth is usually achieved in pure continuous cultures by control of turbidity (14) or by limiting quantity of essential nutrients(12,13). However, this type of control is difficult to attain in the case of mixed populations in continuous culture.

Thus, the exact status of the organisms multiplying in mixed continuous cultures cannot be precisely defined. Nevertheless, the situation is probably more truly representative of their status in their respective ecological niches than is a static situation in which organisms reach a maximum stationary phase as a consequence of a complete ex-

TABLE III. Effect of Prior Inoculation with Enterococci on Mortality of Embryonated Eggs Infected with *V. cholerae*.

V. cholerae inoculum (log)	Mortality* Control	Enterococci†
10^0	1/8	0/8
10^1	8/8	4/8
10^2	8/8	3/8
10^3	8/8	2/8
10^4	8/8	3/8
10^5	7/7	2/5

* No. dead/total. 48 hr after challenge.
† 10^6 enterococci inoculated 24 hr prior to cholera challenge.

haustion of nutrients or accumulation of toxic end-products of the multiplying cells themselves.

As demonstrated above, certain interactions occur between the bacterial species we have studied which are not readily demonstrated by other techniques on agar medium. *V. cholerae* grew readily on a lawn of growing enterococcus cells. The reverse was also true. Similarly, no inhibition was noted between adjacent colonies of one or the other organism. The technic of Fredericq(22) revealed no antagonism. No antibiotics against *V. cholerae* were demonstrable in 24 hour filtrates of enterococcus cultures. At present, there is no explanation of these effects. It seems unlikely that the turnover rate of 100% during at most 4 generations of the growing enterococcus could permit the latter to reduce significantly nutrient materials available to the vibrios. Elimination of this possibility, however, awaits further study. It appears more likely that suppression of cholera vibrios by enterococci in continuous mixed culture is due to the momentary existence of intermediates of the enterococcus metabolism which are inhibitory to the vibrios.

Summary: An apparatus for studying interactions of bacteria in continuous culture has been described. It was shown that, in the dynamic situation of continuously renewed nutrient, growth of *V. cholerae* is partially suppressed in the presence of growing enterococci and lactobacilli, and that the inhibition was due largely or entirely to the enterococcus and took place under both aerobic and anaerobic conditions.

The fundamental cause of the inhibition of cholera vibrios by enterococci is not known, but is not due to the inhibitory effect of variation of hydrogen ion concentration, and probably cannot be attributed to competition for nutrients.

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