ECOLOGICAL FACTORS INFLUENCING THE RELATIONSHIPS BETWEEN KLEBSIELLA AND SHIGELLA IN MIXED CULTURE

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ABSTRACT

HENTGES, DAVID J. (Loyola University, Chicago, Ill.), and MACDONALD FULTON. Ecological factors influencing the relationships between Klebsiella and Shigella in mixed cultures. J. Bacteriol. 87:537–535. 1964.—Viable-cell counts of Shigella flexneri and Klebsiella (Aerobacter aerogenes) in pure and mixed culture were made during growth under predetermined conditions of temperature, pH, oxygen supply, and nutrient supply. In pure culture, environmental changes had marked effects on the Shigella populations. Klebsiella populations were not affected except at 44°C or when aerated; under these conditions, the populations were smaller. In nonaerated mixed culture, under different conditions of temperature, pH, and nutrient supply, Klebsiella interfered with the multiplication of Shigella. Exponential growth of Shigella was interrupted at about the time Klebsiella populations attained a maximal size. In contrast, the presence of Klebsiella in an aerated mixture had little or no effect on Shigella multiplication because Klebsiella failed to attain a maximal population. Different environmental conditions resulted in different Klebsiella to Shigella ratios in mixed cultures. When conditions were favorable for Shigella multiplication, as shown by pure culture controls, the proportion of Shigella in the mixture was generally greater than when conditions were unfavorable.

The majority of studies dealing with the effects of environment on bacterial population growth have been limited to pure cultures of bacteria. Numerous papers on this subject were summarized by Williams and Spicer (1957) and Meynell and Goodner (1961). In mixed cultures, the emphasis has been on the interactions between the bacteria (Waksman, 1945). The outcome of population growth has often been interpreted as the direct effect of one organism on another. Bowing and Wynne (1951), for example, attributed the antagonistic activity of Aerobacter in mixed culture to a direct effect involving close contact of Aerobacter cells with cells of the inhibited species. Later, Wynne and Norman (1953) broadened their interpretation of antagonism to include, in this case, indirect effects operating through a hypothetical metabolic product.

In the ecology of higher plants and animals, antagonism, such as predatory behavior or parasitism, is of comparatively little importance. The external environment, on the other hand, plays an important role in the ecology of these forms. With respect to microorganisms, relatively little is known concerning the effects of environment on the growth of mixtures of bacteria. Freret (1962) presented evidence to show that Escherichia coli, A. aerogenes, and Proteus vulgaris inhibited Shigella flexneri in broth cultures kept in an oxygen-free atmosphere. Inhibition was reversed by aeration or by the addition of glucose to the medium. Freret’s work demonstrated the effect of environmental change on mixtures of bacteria. In general, however, attempts have not been made to differentiate between “organism on organism” effects and effects brought about solely by changes in the environment in which the mixtures are growing. No studies exist of bacterial associations in which measurements are made of the influence of systematic alterations in the environment.

In this paper, we describe experiments designed to examine the influence of selected environmental changes on the relationships between Klebsiella and S. flexneri in mixed culture. Measurements were made of the effects of different conditions of temperature, pH, oxygen supply, and nutrient supply on the popu-
lation equilibrium between the organisms multiplying in vitro.

**Materials and Methods**

**Microorganisms.** The Shigella and Klebsiella (*Aerobacter* 23) strains were used in earlier studies (Hentges and Freter, 1962). They were chosen because of an antagonistic effect of Klebsiella on Shigella. The Shigella strain conformed to the biochemical scheme outlined for *S. flexneri*, type 2, by Edwards and Ewing (1962), except that it failed to produce indole. The Klebsiella strain liquefied gelatin, but otherwise conformed to the biochemical scheme of Edwards and Ewing (1962) for Klebsiella. The Illinois Department of Public Health identified the Shigella strain serologically as *S. flexneri*, type 2a. The serotype of the Klebsiella strain was not determined.

**Synthetic medium.** The organisms were grown in a liquid synthetic medium composed of 0.1% L-glutamic acid, 0.1% glucosamine hydrochloride, 0.6% Na$_2$HPO$_4$, 0.15% KH$_2$PO$_4$, 0.5% NaCl, and 0.001% nicin. Experiments showed that the Shigella strain required nicin as an accessory growth factor. Because of a deleterious effect in autoclaving glucose with phosphate, the glucosamine was prepared as a separate solution and, after autoclaving, combined aseptically with the other constituents of the medium. The final pH of the medium was approximately 7.0 without adjustment.

**Plating methods.** The flood plate method (Hentges and Fulton, 1960) was used for enumeration of pure cultures of *Shigella* and *Klebsiella* and for differential counting of the two strains in mixed culture. Veal Infusion Agar was used for enumeration of *Klebsiella* in pure and mixed culture. A specially prepared agar medium, VIV agar, was used for enumeration of *Shigella*. To prepare VIV agar, 1% glucose was added to Veal Infusion Agar. The medium was autoclaved twice. Before pouring plates, vionycin sulfate was added to give a final concentration of 0.4 mg/ml. This medium prevented the growth of *Klebsiella* colonies but permitted development of *Shigella* colonies.

**Inoculum.** To insure an inoculum that would give reproducible results, a VIV agar plate was streaked with a stock culture of *Shigella*. The plate was incubated for 48 hr at 37°C. From 10 to 20 isolated colonies were picked from the plate into a tube containing 10 ml of synthetic medium. The tube was incubated for 18 hr in a 37-C water bath. After incubation, a 10-fold and then a 100-fold dilution was made of the culture in synthetic medium. The 10$^{-4}$ dilution contained approximately 10$^4$ organisms per ml.

A stock culture of *Klebsiella* was streaked onto a Veal Infusion Agar plate. The plate was incubated for 24 hr at 37°C. Portions of about five isolated colonies were picked from the plate into a tube containing 10 ml of synthetic medium. The tube was incubated for 18 hr in a 37-C water bath. After incubation, the culture was diluted in synthetic medium, 3-fold, then 100-fold, and then again 100-fold. The final dilution contained approximately 10$^4$ organisms per ml.

These inocula were added so that the initial count for the organisms in all the experiments was approximately 10$^8$ organisms per ml.

**Standard conditions of cultivation.** Under standard conditions, the organisms were grown in synthetic medium of pH 7 in a 37-C water bath. Culture tubes were plugged with cotton and covered with loose aluminum caps.

**Temperature alterations.** The culture tubes were placed in water baths adjusted to various temperatures. Water-bath temperatures did not fluctuate more than ±0.5°C during the experiments.

**Alterations of pH.** Adjustments in pH were made simply by adding NaOH or HCl to the synthetic medium until the desired pH was attained. All pH measurements were made with a Coleman Metrion pH meter.

**Alterations in oxygen supply.** Control determinations were made under stationary conditions in an air incubator set at 37°C. Cultures to be aerated were placed in a “Migitator” (Elmac Engineering Co.), which rotated tubes in a horizontal position at 25 rev/min inscribing a circle with a 3.5-in. diameter. The apparatus was put in an air incubator set at 37°C. For conditions of reduced oxygen supply, tubes containing 10 ml of synthetic medium were placed in a boiling-water bath for 10 min to remove dissolved oxygen. Tubes were then placed in an ice bath for about 1 min to cool. When cool, they were inoculated. Immediately after inoculation, a 1-cm layer of melted petrolatum was poured on the surface of the liquid medium. The efficacy of this method for reducing oxygen supply was tested by methylene blue reduction.
Control tubes containing 0.002% methylene blue become colorless after 10 min in the boiling-water bath, indicating reduced conditions in the medium. Cooling and inoculating caused slight reoxygenation of the medium, as shown by development of a light-blue color. The color disappeared after a few hours in the incubator.

Alteration of nutrient concentration. Culture medium of standard strength was designated as 1X. In dilute medium, designated as 0.1X, the concentration of the organic constituents, glucosamine, glutamic acid, and niacin, was reduced to 0.1 times standard concentration. In concentrated medium designated as 10X, the concentration of organic constituents was increased to 10 times standard concentration, and NaCl was eliminated to minimize the increased molarity over the standard.

Determination of population size. At 5, 9, 27, and 53 hr, samples were removed from the cultures and dilutions were made with saline when required. A 1-ml portion of Shigella culture or saline dilution was flooded on the surface of a dried VIV agar plate. Klebsiella cultures were flooded on the surface of dried Veal Infusion Agar plates. Mixed cultures were flooded on VIV agar plates to count Shigella colonies and on Veal Infusion Agar to count Klebsiella colonies. VIV plates were incubated 48 hr to ensure sufficient development of Shigella colonies. Colonies were counted with an electronic colony counter (model C-100, New Brunswick Scientific Co., New Brunswick, N.J.). Temperature and oxygen experiments, run in duplicate, were repeated four times. Nutrient and pH experiments, also run in duplicate, were repeated three times.

RESULTS
The actual results consist of viable counts of pure and mixed cultures of Shigella and Klebsiella. The counts were done in duplicate at four culture ages. Four environmental factors were tested, each at three levels. The experiments were repeated several times. Analysis of the large number of counts obtained indicated that the population sizes at 27 hr of growth suitably illustrated the effects of the environmental factors studied on the multiplication of the organisms. Table 1 gives the 27-hr populations

TABLE 1. Mean viable Shigella and Klebsiella population sizes at 27 hr*

<table>
<thead>
<tr>
<th>Environmental condition</th>
<th>Shigella</th>
<th></th>
<th></th>
<th>Klebsiella</th>
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<tbody>
<tr>
<td></td>
<td>Pure</td>
<td>Mixed</td>
<td>Pure</td>
<td>Mixed</td>
<td>Pure</td>
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<td>Incubation temperature</td>
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<tr>
<td>(pH 7, static, 1.0X nutrient conen)</td>
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<tr>
<td>30°C</td>
<td>4.25 × 10⁴</td>
<td>6.17 × 10⁴</td>
<td>3.32 × 10⁴</td>
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<tr>
<td>37°C</td>
<td>6.70 × 10⁴</td>
<td>1.37 × 10⁴</td>
<td>3.46 × 10⁴</td>
<td>3.16 × 10⁴</td>
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<tr>
<td>44°C</td>
<td>2.00</td>
<td>7.50</td>
<td>7.27 × 10⁴</td>
<td>9.57 × 10⁴</td>
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<td>pH of medium</td>
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<td>(37°C, static, 1.0X nutrient conen)</td>
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<tr>
<td>6.0</td>
<td>6.50 × 10⁴</td>
<td>1.32 × 10⁴</td>
<td>2.72 × 10⁸</td>
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<td>7.1</td>
<td>6.02 × 10⁴</td>
<td>1.09 × 10⁴</td>
<td>1.80 × 10⁸</td>
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<td>8.0</td>
<td>6.58 × 10⁴</td>
<td>1.05 × 10⁴</td>
<td>2.90 × 10⁸</td>
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<tr>
<td>Aerated</td>
<td>2.75 × 10⁴</td>
<td>1.53 × 10⁴</td>
<td>9.23 × 10⁴</td>
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<td>Static</td>
<td>5.59 × 10⁴</td>
<td>8.41 × 10⁴</td>
<td>1.83 × 10⁸</td>
<td>2.64 × 10⁸</td>
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<tr>
<td>Reduced</td>
<td>1.49 × 10⁴</td>
<td>8.52 × 10⁴</td>
<td>1.48 × 10⁸</td>
<td>1.38 × 10⁸</td>
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<td>Nutrient concentration</td>
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<tr>
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<tr>
<td>0.1X conen.</td>
<td>4.58 × 10⁴</td>
<td>2.10 × 10⁸</td>
<td>2.73 × 10⁸</td>
<td>1.95 × 10⁸</td>
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<tr>
<td>1.0X conen.</td>
<td>1.29 × 10⁴</td>
<td>1.85 × 10⁴</td>
<td>3.62 × 10⁴</td>
<td>2.73 × 10⁸</td>
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<tr>
<td>10X conen.</td>
<td>1.24 × 10⁴</td>
<td>1.45 × 10⁴</td>
<td>4.95 × 10⁴</td>
<td>3.75 × 10⁸</td>
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</table>

* All figures represent averages of six or more counts.
† Standard control conditions.
of *Shigella* and *Klebsiella* in pure and in mixed culture. To easily visualize population differences, population sizes are represented with bar graphs in Fig. 1. Since *Klebsiella* counts were always greater than *Shigella* counts, the larger stippled bars representing *Klebsiella* populations are drawn behind the shorter shaded bars which represent *Shigella* populations. The upper row of bar graphs in the figure show the population sizes of the two organisms multiplying in pure cultures. Stippled bars indicate that *Klebsiella* counts were from $10^8$ to $4.0 \times 10^9$ cells per ml of culture medium under almost all the conditions of temperature, pH, oxygen supply, or nutrient supply. There were two notable exceptions: when *Klebsiella* cultures were incubated at 44 C and when cultures were aerated at 37 C. At 44 C, the mean viable count dropped to $7.3 \times 10^3$ cells per ml; when aerated, the count dropped to $0.2 \times 10^6$ cells per ml. The shaded bars in the upper portion of Fig. 1 show that *Shigella* populations varied considerably with changes in environment. Under standard conditions, the 27-hr populations were in the neighborhood of $10^9$ cells per ml of medium. At 44 C, the average count was only two cells. Other low counts were recorded at 30 C, in medium at pH 6, in anaerobic medium, and in dilute medium (0.1 X). Populations were equivalent to standard in medium at pH 8 and in concentrated medium (10X).

The lower row of bar graphs in Fig. 1 represents population sizes of the organisms multiplying in mixed culture. As with pure cultures, *Shigella* populations varied with environmental
changes. Under standard conditions, *Shigella* population sizes were about $10^4$ cells per ml. Low counts were recorded at 30 and 44 C, at pH 6, under static or anaerobic conditions, and in dilute medium. Counts were highest in concentrated medium and when cultures were aerated. *Klebsiella* population sizes, as with pure cultures, were from $10^6$ to $4.0 \times 10^6$ cells per ml except when the cultures were aerated or incubated at 44 C. Under these conditions, the populations were smaller.

The influence of mixed culture growth on population sizes can be examined by comparing corresponding bar graphs for the organisms in pure and mixed culture. In Fig. 1, the bar graphs for *Klebsiella* in the upper portion of the figure are almost identical with the bar graphs in the lower portion of the figure. *Klebsiella* populations were about the same size in pure and mixed culture. A comparison of the bar graphs for *Shigella* in Fig. 1 shows that the overall pattern of bar heights is similar in the upper and lower portions of the figure, but the bars are generally shorter in the lower portion of the figure. *Shigella* populations were smaller in mixed culture than in pure culture with two exceptions. When cultures were aerated or when they were incubated at 44 C, the *Shigella* populations in pure and mixed culture were nearly the same.

Typical growth curves for *Shigella* and *Klebsiella* in pure and mixed culture under standard conditions are illustrated in Fig. 2. The solid line represents growth curves for *Klebsiella* in both pure and mixed culture. The points from which pure and mixed culture curves were plotted were so close that both are represented by a single line. *Klebsiella* curves show that the multiplication rate, the length of the exponential phase, and the maximal population for *Klebsiella* were the same in pure and mixed culture. The broken lines represent growth curves for *Shigella* in pure and mixed culture. In the early stage of the growth cycle, pure and mixed culture curves are identical, suggesting that multiplication rates were the same. The curves show that the exponential phase was interrupted in mixed culture and that its maximal population size was smaller than in pure culture. The dotted lines were obtained by extrapolating the growth curves in a manner based on the interpretation of growth curve forms by Buchanan (1918). These lines show that the exponential phase for *Shigella* in mixture was interrupted at about the same time the *Klebsiella* strain attained a maximal population.

The populations for *Shigella* were nearly the same in pure and mixed culture when cultures were aerated or when they were incubated at 44 C. Figure 3 shows the growth curves for *Shigella* and *Klebsiella* at 44 C. The broken lines,
representing the *Shigella* curves, indicate that *Shigella* failed to multiply at 44°C. Death rates were similar in pure and mixed culture, hence the similarity of pure and mixed culture populations. The single unbroken line represents both pure and mixed culture *Klebsiella* curves. The *Klebsiella* multiplication rate was considerably reduced when compared with its rate under standard conditions. Figure 4 shows the growth curves for the organisms under conditions of aeration. The curves illustrate that multiplication rates for both *Shigella* and *Klebsiella* were reduced when compared with standard conditions, but that the effect was more pronounced.

**FIG. 3.** Growth curves of *Shigella* and *Klebsiella* in pure and mixed culture at 44°C (pH 7, static, 1.0X nutrient concentration).

**FIG. 4.** Growth curves of *Shigella* and *Klebsiella* in pure and mixed culture under conditions of aeration (37°C, pH 7, 1.0X nutrient concentration).
with Klebsiella. Because of its reduced multiplication rate, the Klebsiella strain failed to attain a maximal population in the time limit for the experiments. It is noteworthy that, under these conditions only, exponential growth of Shigella was not interrupted in mixed culture. The growth curves for Shigella in pure and mixed culture were similar under aerated conditions only.

The population equilibrium established between Shigella and Klebsiella in mixture varied considerably under the different conditions of environment. Table 2 gives the Klebsiella-Shigella ratios at 27 hr under different conditions of environment. The ratio between the organisms was greatest under static and reduced conditions and when the medium was at pH 6. The ratio was smallest under conditions of aeration, when Shigella multiplied without interference in mixture, and when Klebsiella failed to attain its usual population maximum.

**Discussion**

The experimental evidence indicates that environment greatly influences the population equilibrium between Klebsiella and Shigella. Table 2 shows, in the aeration experiments, that the equilibrium ranged from a ratio of 50:1 to a ratio of 3,100,000:1. In other words, a 62,000-fold difference in the equilibrium ratio occurred as the result of the alteration of a single known factor in the environment, the oxygen supply. Table 1 shows that Klebsiella population sizes were relatively constant under the environmental conditions employed. Differences in Klebsiella-Shigella ratios were due primarily to differences in Shigella population sizes in mixed culture under the different environmental conditions. In general, if Shigella grew well in mixed culture, it also grew well in pure culture. At pH 7, for example, the Shigella population in mixed culture was 1.09 × 10^6 cells per ml, and in pure culture 6.02 × 10^6 cells per ml. At pH 6, on the other hand, the Shigella population was only 1.32 × 10^6 cells per ml in mixture and 6.50 × 10^6 cells per ml in pure culture. Shigella populations at pH 7 were larger than at pH 6 in both pure and mixed cultures. Klebsiella populations were about the same at pH 6 and pH 7. Examination of mixed cultures alone would suggest that inhibition of Shigella by Klebsiella was greater at pH 6 than at pH 7. Pure culture controls were necessary to establish that, in mixture at pH 6, the smaller Shigella population was due to an environmental effect rather than an "organism on organism" effect. This illustrates the importance of analyzing the influence of environmental factors when drawing conclusions about the relationships between organisms.

The experiments show that it is possible to regulate the equilibrium ratio between the organisms to some extent by adjusting the environment. On aerating the medium, for example, the Klebsiella-Shigella ratio was reduced from 3,100,000:1 under stationary conditions to 50:1. Information of this nature may have utility in the practical problem of isolating Shigella from the feces. In the past, unsuccessful attempts to enrich Shigella growth have been based on adjustment of nutritional factors in the enrichment medium. Perhaps a systematic alteration of environmental factors would prove to be a more effective approach to the problem. It is conceivable that environmental conditions exist which favor Shigella growth over Klebsiella or E. coli growth. To test this hypothesis, the experiments described here would necessarily have to
be extended to include a broad range of environmental factors.

The evidence indicates that the Klebsiella strain is a harder organism than the Shigella strain in several ways. No matter how the environmental factors under study were varied, the Klebsiella multiplication rate was greater than the Shigella multiplication rate. Without exception, Klebsiella multiplied without interference from Shigella in mixture. When antagonism occurred, the Klebsiella strain was the antagonistic species.

The mechanisms of Klebsiella antagonism are not clearly understood. In mixture, Shigella multiplication was always interrupted at the onset of the Klebsiella stationary phase (Fig. 2). In one instance, when Shigella multiplied without interference from Klebsiella (Fig. 4), a stationary phase for Klebsiella did not occur. These results suggest that the mechanisms responsible for Shigella antagonism are also responsible for the Klebsiella stationary phase.

The stationary phase has been attributed at one time or another to exhaustion of essential nutrients (Monod, 1942; Van Niel, 1944), accumulation of acid (Cohen and Clark, 1919), attainment of "M-concentration" (Bail, 1929), accumulation of toxic products (Chesney, 1916; McLeod and Gordon, 1922), and depletion of oxygen (Rahn and Richardson, 1942). Any one of these factors, or a combination, may be responsible for the concomitant cessation of Klebsiella and Shigella growth in culture medium. It does not seem likely that nutrient depletion was responsible. According to Monod (1942), the maximal population size is proportional to whatever nutrient is limiting in the medium. Klebsiella and Shigella pure culture populations were approximately the same size in 1X and 10X media (Table 1). This indicates that nutrients were not limiting at these concentrations. Multiplication of both strains ceased in what appears to be a nutrient excess, suggesting that some other mechanism was responsible for growth cessation. Acid accumulation is an unlikely possibility. Klebsiella and Shigella multiplication at pH 7 was almost identical with multiplication at pH 8. When multiplication stopped in medium at pH 8, the pH of the medium had dropped to a value no lower than 7.5. From this, it appears that mechanisms other than acid accumulation or nutrient depletion were responsible for concomitant cessation of growth.

Von Wikulil (1932) stated that the sum of the population sizes of the species in mixed culture never exceeds the greatest population size attained by either of the species in pure culture. In other words, the sum of the "M-concentrations" in mixed culture is never greater than the largest "M-concentration" for one of the species in pure culture. This theory is difficult to prove or disprove unless the populations of the species in mixture are of the same magnitude. In these experiments, Klebsiella and Shigella populations were not of the same magnitude. The margin of error in determining the mean Klebsiella count in mixture was often greater than the total Shigella count. It is, therefore, impossible to evaluate the relationship between Klebsiella and Shigella in terms of "M-concentration."

Accumulation of toxic substances is another theory proposed to account for the stationary phase. It seems improbable that toxic substances were responsible for the concomitant cessation of Klebsiella and Shigella growth. Shigella grew out in supernatant fluids from Klebsiella broth cultures which had been incubated both for 24 and 48 hr. Furthermore, Freter (1962) showed that substances toxic to Shigella could not be demonstrated in broth culture filtrates of Aerobacter, E. coli, or Proteus, although these strains were antagonistic to Shigella in mixed broth culture. When Shigella was inoculated into culture filtrates, there was no decrease in its growth rate or population size as compared with fresh broth. Apparently, toxic substances did not cause the antagonism in the mixed cultures.

Freter's results showed that, in mixed cultures, antagonistic strains strongly reduced the culture medium and then successfully competed with Shigella for fermentable carbohydrates. In the experiments described in this paper, it is possible that fermentative metabolism had proceeded to completion at the time Klebsiella attained a population maximum and Shigella growth ceased. Dagley, Dawes, and Morrison (1951) aerated a culture of Aerobacter aerogenes which had attained a population maximum under nonaerated conditions. Aeration increased metabolism and caused reinitiation of cell multiplication. Perhaps aeration of mixed Klebsiella and Shigella cultures, when growth ceases, would
cause reinitiation of multiplication of both strains and permit Shigella to attain a population size equivalent to its population in pure culture. Additional work is needed to test this hypothesis.

Ecological principles are being applied with increasing frequency to pure culture bacterial population studies (Williams and Spicer, 1957; Meynell and Goodner, 1961). In nature, bacteria usually exist as mixed populations. This investigation represents the application of ecological principles to a mixture of bacteria. The results illustrate the importance of the analysis of environmental effects on mixtures of bacteria when the outcome of population growth is being investigated. Mixed bacterial population studies can be approached effectively from an ecological viewpoint, i.e., an examination of the relationships between organisms and an analysis of the effects of environment on these relationships.

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Literature Cited


