INTERACTIONS OF CERTAIN ORAL MICROORGANISMS AS MEASURED BY CONTINUOUS FLOW SYSTEMS.

by

Bente le Fevre Simonsen, D.D.S.

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APPROVED

[redacted]

R. B. Parker
Associate Professor of Bacteriology

[redacted]

Ellis B. Jump, D.M.D., Ph.D.
Chairman, Graduate Education Committee

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INTRODUCTION

The oral microflora is comprised of a mixture of incredible complexity which has thus far defeated even crude evaluation. Very little is known about factors leading to the development and maintenance of the flora and even less is understood about microbiological factors involved in dental diseases. In either event one is faced with the problem of measuring the net effect of a mixed microbial population and determining the contribution of the individual species within the group.

The problems of immediate interest in the study of interactions of microorganisms are those indigenous to man, as they interact with each other, pathogens, and transient saprophytes. It is possible that these interactions between members of a complex microbial flora could be critical determinants of the delicate biochemical balance between these microorganisms and the tissues of the host, the status of which determines the relative state of health or disease. A number of investigators have suggested that interactions between oral species in mixed microbial populations are pertinently related to actinomycosis and periodontal disease with special reference to fusospirochetal infections of man.

However, it has not been possible to establish their etiology on a specific basis so that the agent in turn could be studied by the usual bacteriological methods. It is the purpose of this study to approach the problem of mixed infections by an "in vitro" system of mixed cultures which will allow quantitation of the interactions between three bacterial species normally listed as members of the oral flora in terms of generation time.

REVIEW OF LITERATURE

Since this project is based upon the maintenance of organisms in a steady state of growth by continuous culture methods before admixture in a common cell, the literature is reviewed only in respect to the development of continuous culture techniques necessary to accomplish this request. By definition a continuous culture is one that is maintained and regulated by the constant flow of nutrient through a growth cell.

Before the beginning of microbiology as a science the art of fermentation as early as 1670 (25) used the principle of continuous culture in production of vinegar and in the production of yeast by 1879 (43). A number of papers and patents subsequently appeared dealing with this fermentation process which has the widest application today in the industrial production of antibiotics, yeast for brewing and baking, and alcohols. In contrast, the application of continuous culture technique as an experimental tool in microbiology dates for the most part from 1945. There are scattered references prior to that date which show a lack of comprehension of the basic principles of open systems in which growth can be regulated by rate of flow of medium or concentration of nutrient. Thus, Utenkov (53) in 1941 dating his work back to 1922 started experimental work on the assumption that the batch method of studying pure bacterial cultures did not bring out the true characteristics of microorganisms or allow sufficient regulation of the development of cultures, while Moyer (29) in 1959 devised a continuous culture system to obtain bacteria in sufficient bulk for chemical analysis. The possible use of continuous culture as a promising way to investigate mixed cultures was proposed by Rogers and Whittier in 1930 who found a continuous culture method

was better than the static method of batch culture for studying the reproductive capacity of bacteria. Unfortunately, they were unable to regulate the numbers of each species in a mixture.

From this time until 1950 most of the interest in bacterial growth was in the kinetics of cultures growing in the usual test tube or closed system. This material has been adequately reviewed by Monod (27). Of the four major divisions of a growth curve (lag, logarithmic, stationary and senescence) the logarithmic or exponential growth rate has proved the most important in respect to continuous culture because it is here where the clearest definition and reproductibility for growth and physiological studies can be attained. Furthermore, the rate of reproduction is constant in this period and can be calculated as the generation time (θg) for that particular culture under the conditions specified. It is based on the fact that under ideal and unrestricted conditions for the logarithmic phase, division of cells occurs at regular intervals, so that a simple mathematical expression of growth can be developed in which:

$$X^\circ_2 = 2^n X^\circ_1 \qquad (1)$$

X°_1 being the initial number of cells, X°_2 the final number of cells, and n the number of times the division has occurred.

This may be further developed if the initial and final populations are known, so the number of doublings (generations) that have occurred during the experiment can be computed by:

$$2^n = \frac{X^\circ_2}{X^\circ_1}$$

$$n \log 2 = \log \frac{X^\circ_2}{X^\circ_1}$$

$$n = \frac{\log X^\circ_2 - \log X^\circ_1}{\log 2}$$

If Θg is the time for one generation, then n generations will require ($n\ \Theta g$) which is also the total time of the experiment, so that:

$$n \times \Theta g = t \qquad (2)$$

By substituting for n the generation or division time may be obtained directly:

$$\Theta g = \frac{t}{n} = \frac{t \log 2}{\log X^\circ_2 - \log X^\circ_1} \qquad (3)$$

Another general equation for growth in the logarithmic phase of growth in a batch culture is the following:

$$\frac{dX}{dt} = kX$$

where X represents the number of cells per ml, k equals a growth constant and t the time. By integration this will lead to:

$$X^\circ_2 = X^\circ_1 e^{kt} \qquad (4)$$

from (1) and (4):

$$X^\circ_1 2^n = X^\circ_1 e^{kt}$$

$$2^n = e^{kt}$$

$$n = \frac{kt}{\ln 2} \qquad (5)$$

from (2) and (5) the following is derived:

$$k = \frac{\ln 2}{\Theta g} \doteq \frac{0.694}{\Theta g}$$

Thus the growth rate constant k can be computed for each single bacterium growing in a certain medium having a generation time Θg.

It is well known that if one wants to keep a culture in logarithmic phase one has only to transfer it during that phase, but this would have to be repeated at approximate six-hour intervals, a problem which can be solved by having fresh nutrient pass through the system. This is a continuous culture. Thus, Monod described the bactogene (1950) (28), Novick

and Szilard (34, 35) the chemostat, Fox and Szilard (5) a system to grow bacteria under steady state conditions and Northrop (32, 33) the turbidostat. Jerusalemskij (12) has pointed out that if density is measured, only part of the total number of cells continue to multiply and contribute to the increase in turbidity. This, however, is only true if the investigator uses aging cultures. Turbidity is valid as a measurement of growth of actively growing bacteria in a steady state as in the turbidostat. Advancement of the theory resulted in the production of new and better laboratory equipment of various types. As a fine example of highly developed apparatus for continuous flow of nutrients, even for the most fastidious microorganisms, reference should be made to the system of Heden, Holme and Malmgren (9).

The maintenance of a constant environment is an obligatory requirement for the establishment of balanced growth in a bacterial culture. This is easily accomplished in a continuous culture device where the renewal of the medium is constantly ensured. Here the exponential part of the growth curve for bacterial growth becomes significant because only then does uniform multiplication take place. Malek (22) Kjeldgaard (16), Malmgren and Heden (23) also point out that continuous flow systems meet the optimal requirements of fast vegetative multiplication of microorganisms. Therefore, the phenomena which are associated with the multiplication of bacteria can be studied most conveniently under continuous or steady state conditions which are defined as follows:

a) Continuous Flow System is a system where a continuous flow of fresh nutrients and continuous withdrawal of used modified materials from the culture gives good conditions for growth.

b) Steady state is a bacterial population characterized by its constancy. In the present study it was obtained by a predetermined flow of medium containing a limiting factor.

Continuous culture methods were laid on a solid, theoretical basis, particularly due to the mathematical treatment of the chief growth principles in open systems by Monod (27, 28), Spicer (51), Powell (40, 41) and Gerhardt and Bartlett (6). Novick (36), Malek (22) and James (11) evaluated their system from the view of experimental possibilities and perspectives which were published in extensive papers of a more general character devoted to the questions of continuous culture methods. The theory has been further developed in a number of other publications. Thus, Herbert, Elsworth and Telling (10) undertook a mathematical analysis of the kinetics of bacterial growth and changes in the concentration of microorganisms by means of a minimum number of simple postulates. They especially considered the steady state problem and the effect of various rates of dilution at various substrate concentrations in the inflowing medium. Their theoretical conclusions were supported by practical results. Powell (40, 41) analyzed the relationship between the rate of growth and generation times in cultures growing under continuous flow conditions. He discussed the various factors leading to the establishment and preservation of a steady state.

Mathematical treatment of open systems. Open systems lend themselves to mathematical treatment, since the rate of transfer of reacting materials into the system is commonly either constant or periodic and in turn the products are continuously or periodically withdrawn.

A continuous culture starts in the same fashion as a batch culture, then some time after inoculation usually in the exponential phase the continuous flow of medium is started. A constant volume V is maintained

in the growth tube by feeding the system at a fixed rate F and withdrawing the overflow at the same rate.

The generally applicable equation for the system is:

Input + Growth = Output + Accumulation.

$$FX_1 + kX_2V = FX_2 + V\frac{dX}{dt} \qquad (6)$$

where X_1 equals the number of organisms entering the system and X_2 equals the number of cells withdrawn from (6):

$$F/V\ X_1 + k\ X_2 = F/V\ X_2 + \frac{dX}{dt}$$

$$X_2\ (k - F/V) + F/V\ X_1 = 0 \qquad (7)$$

for n being the number of generations we have:

$$n = \frac{\log X_2 - \log X_1}{\log 2} \qquad (8)$$

and from equation (3):

$$\Theta g = \frac{t \log 2}{\log X_2 - \log X_1}$$

From equation (7) it can be seen that when the loss of cells through overflow (X_2 F/V) is greater than the increase of cells by growth (X_2 k) the number of cells eventually will decrease to zero. If the cell increase (k X_2) is greater than (X_2 F/V), the number of cells will continue to increase until the utilization of the limiting nutrient of growth factor in the medium determines the growth rate.

Open systems contra closed systems. As nutrients in a batch culture are exhausted and metabolites accumulate, the rate of the process is decreased until it reaches the point when no free energy is transformed. At this point the process stops and an equilibrium is established. This is characteristic of a closed system. So far microbiologists have almost exclusively been concerned with processes in closed reaction systems

leading to equilibrium. However, the bacterial cell as a whole is not a closed system, it is never in a true equilibrium but in a steady state.

A system is closed if no material enters or leaves it. It is open if there is import and export and thus exchange of components. Living cells fundamentally are open systems, maintaining themselves in exchange of materials with environment and in continuous building up and breaking down of their material components. The concept of an organism as an open system has been advanced to its greatest extent by von Bertalanffy (55). A closed system, he points out, must according to the second law of thermodynamics, eventually reach a time-independent equilibrium with maximum entropy and minimum free energy. An open system may attain a time-independent state where the system as a whole remains constant, though there is a continuous flow of its components. This is what can be defined as a steady state. In order to perform work a system must not be in equilibrium but tending to attain it. This means that the system must be open in order to maintain a steady state. In a precisely defined steady state the application of results from open systems is facilitated and the mathematical interpretation of the individual kinetic reactions is simplified.

The continuous culture system has a number of advantages over the batch process, some of which are listed:

1) The course of reactions can be studied in any chosen phase of the process under constant steady state conditions in which the time factor is eliminated. The influence of nutrients, temperature, pH, stirring, aeration and other factors on the course of reactions can be determined easier and without complications due to the incessantly changing conditions.

2) The mathematical treatment of the process is simpler.

3) There is a greater possibility of automating the whole process and of regulating it by a suitable application of physico-chemical methods.

4) The production of bacterial cells or bacterial products is more efficient due to two factors: the first, dimensions of a given apparatus can be decreased while the same production rate is preserved, and second, the culture is in a more active physiological state than a batch culture.

Types of continuous flow systems. Continuous culture systems can be divided into externally and internally controlled systems:

a) Externally controlled systems are those where the steady state growth rate is kept below the maximum by feeding a low concentration of some required nutrient. This principle has been used in the chemostat and in the bactogene;

b) Internally controlled systems are regulated by controlling the population level, which is normally accomplished by a photocell reading of the optical density. Northrop (32, 33) has been using this type of control in his continuous culture apparatus and so have Fox and Szilard (5) in their turbidostat.

In brief, the difference between the two systems is that in the externally controlled systems the feeding rate is chosen by the investigator and the microbial population finds its own level according to the flow of limiting nutrient, while in the internally controlled continuous flow systems the bacterial population is fixed and the flow rate finds its level according to the chosen turbidity of the culture under study. Also it should be noted that in externally controlled systems the organisms are starved, in the internal systems they are not.

In the internally controlled systems a major difficulty has been to avoid adherence of bacteria to the walls of the growth tube. Although some attempts have been made to prevent this (32), such measures have never been successful over long periods of time. This is not a problem in an externally controlled type of continuous flow apparatus.

Powell (40, 41) pointed out that there is no essential difference between the two systems; that is, with the chemostat or bactogene the experimenter fixes the flow rate, and therefore the growth rate, as the population level adjusts to the input nutrient concentration. In the internally controlled systems of Myers and Clark (30), Bryson and Szybalsky (3) and Fox and Szilard (5) the experimenter selects the population density and the flow rate adjusts itself to the growth rate as it, in turn, is a function of the medium.

The stability of the steady state in an external system is a function of cellular regulation which makes it inherently better than that of the internal system where the stability is a function of the machinery used in its construction. The better stability of the external system has been demonstrated mathematically by Spicer (51) both for the utilization of growth factors and for the production of growth inhibiting substances. The dependence of growth on the concentration of the limiting growth substances in continuous cultures is an important aspect of these systems. As Novick and Szilard (35) point out, it is not dependent on the concentration of the incoming medium but on the concentration in the culture tube.

For an apparatus planned to operate on the principle of external control it is required that the nutrient flow can be maintained for a long period of time. Monod (28) used a serum pump which employed a rotating

eccentric drum to squeeze continuously a coiled rubber tube to feed the system. The volume of the culture was kept constant at 100 to 400 ml by means of an aspirator tube which sucked liquid from the culture flask whenever the volume exceeded the desired value. Novick and Szilard (34) used injections of medium at short intervals into the growth tube by means of a capillary tube filled with the nutrients to a fixed level by pressure in the reservoir. A homogeneous mixture and aeration was maintained by air bubbling through the chemostat.

When the transfers are continuous the term steady state is applied. If the material transfers are entirely blocked the system becomes closed, reaches equilibrium and dies.

Obviously bath and turbidostat cultures are modifications of the same principle: the growth of bacteria is at a maximum rate in a given medium where this medium imposes no specific restrictions to the growth through limitations of any requirement of the organism. The growth rate as well as other parameters of the bacterial activity under such conditions are determined by a set of interior mechanisms of control. This condition will be called unrestricted growth, in contrast to the limited growth in the chemostat. Under the latter conditions the rate of growth is determined by the actual restricted supply of a specific nutrient and not by the synthetic and regulatory capacities of the cells.

It appears feasible to apply the principles of continuous culture toward measuring the effect of organisms upon each other in a system whereby growth can be controlled in such a way to emulate the oral environment. The first step has been accomplished (38); interactions between two cultures as measured by the effect on generation times calculated for the organisms grown separately and in continuation. This can be

projected toward systems including three or more different bacterial species, which opens up the possibility of investigating some of the complex aspects of oral ecology and infection. That is, none of the infections of the teeth and associated tissues appear to be simple in their etiology. Not only do bacteria combine in complex physiological interrelationships but their pathogenic activity is greatly influenced by imponderable factors in the environment and constitution of the host. Williams and Powlen (57) do point out that the oral milieu can be considered as an open system acting for the oral flora and thus appear to justify the oral environment as a natural chemostate in which the complex is continuously undergoing change with alterations in its chemical and physical characteristics of its parts. Therefore, a deep comprehension of its microbial ecology requires separate study of integral parts such as individual and mixed populations. Such populations may be studied in vivo as well as in vitro in order to obtain better understanding of the numerous factors involved, as emphasized by Williams (58).

Since the original observations by Leewenhoek in 1683, the microorganisms that occur in the oral cavity have been studied in a variety of ways and for numbers of reasons. While these studies have contributed towards a better understanding of the type of microbial flora that may exist in the mouth few attempts have made to determine whether there are ecological relationships between the organisms of particular interest and other forms in the same flora.

MacDonald, Sutton and Knoll (21) and MacDonald and Gibbons (20) have found that while pure cultures derived from mixed periodontal infections in guinea pigs are not infective, combinations of pure cultures obtained from infections may, however, reproduce typical subcutaneous

infections similar to those produced by the total mouth flora. Also other papers by Smith (50), Proske and Sayers (42) and Kestenbaum and Weiss (15) have demonstrated that various combinations of such organisms are infective in mixed cultures. Thus these results provide an example of synergism in the production of disease.

Many of the advantages already mentioned as related to pure cultures in a continuous flow system also apply to the study of microbial mixtures and many investigators have used modifications of the mixed populations. Zubzrycki and Spaulding (65) have employed a modification of the chemostat so that it could be used for long term studies of bacterial mixtures, however, the flow rate in their device cannot be regulated constantly and exactly. Annear (1) used mixed closed system cultures in studying mixed bacterial growth but again the nutritional flow is not continuous permitting the serious objection that the cultures are ageing through the experiment.

With the "drop pair" method Rosebury et al (46) have indicated that Staphylococcus aureus was inhibited by Streptococcus faecalis and that Neisseria catarrhalis was inhibited by S. faecalis. The technique involves cross titration on one plate of two species each at four progressive tenfold dilutions. This technique was only intended as an attempt to find a screening method and it will not make a quantitative evaluation of the interactions possible.

Mergenhagen and Scherp (24) proved that pure cultures isolated from mixed gingival samples gave negative collagenolytic activity but when they were combined they were collagenolytic. Collagenase activity has been demonstrated in material cultivated from the gingival pocked (52, 47, 49) and more from individuals suffering chronical marginal gingivitis than from persons with clinically normal gingivae. The agent causing

these conditions has not as yet been isolated. Thonard and Scherp (52) have demonstrated an inhibition of collagenase activity by the indigenous flora from the gingivae.

Orland and others (37) showed that an "enterococcus" in pure culture or combined with a proteolytic bacillus could produce caries in germ-free rats. Fitzgerald and Keyes (4) could introduce carious lesions in hamsters by oral inoculation of single or pooled cultures of streptococci. Different strains of lactobacilli and diphtheroides also were included in their study single or pooled but they were not able to obtain any significant results for these organisms.

Richardson and Schmidt (44) have by plating technique found inhibition of an oral filamentous organism by S. salivarius, S. mitis and S. epidermidis. The inhibition was ascribed to the acid production by the streptococci and S. epidermidis.

Although in a recent paper Jordan and Keyes (13) could introduce typical disease of the periodontium following inoculation of a gram-positive filamentous organism, this organism had been taken away from the normal environment and from the normal biological equilibrium of the oral cavity.

Lammers (19) further has emphasized that the mouth flora itself maintains a certain antibacterial principle and that the equilibrium also is constant and typical for an individual but varies with different persons. Bjorneso (2) has demonstrated an antibacterial effect of saliva on certain members of the microbiota of the mouth and has ascribed the effect as possibly a result of antagonistic activity of other salivary microorganisms. MacDonald and Gibbons also have discussed the possibility of an existing delicate balance in the oral environment. And Zeldow (62,

63, 64) has reported a bactericidal effect upon L. acidophilus by an antibacterial salivary factor.

Relationships between oral bacteria. Most studies have been done on the Lactobacillus-Yeast relationship. However, none of these studies have used strictly controlled and defined systems. Young and her co-workers (17, 60) have presented evidence of production by oral candida of four of the B-vitamins necessary for growth of several lactobacilli. Wilson and Goaz (59) used a diphasic system where candida was growing in a viscose bag suspended in a flask of an assay broth. The medium could be conditioned by yeast for growth of the lactobacillus, although this is a serious attempt to differentiate and isolate the growth factors produced by the candida for growth of lactobacilli. It must be emphasized that in addition to the above criticism only compounds able to penetrate the viscose membrane can be detected. Furthermore, the factor of dilution by the medium might be of great importance but the system is without any control. The authors did prove that Candida albicans is the active producer of many compounds of importance for growth of lactobacilli but the same factors are present in the saliva which, of course, could originate from the secretions rather than the physiological processes of the yeasts. But this has not been proved and thus far we still have to maintain the theoretical possibility of production of some or all of these growth promoting factors by other oral inhabitants.

Green and Dodd (7) and Green and Inverso (8) have presented evidence of more susceptibility of manitol non-fermenting lactobacilli to the action of streptococci than of the manitol fermenters of this species. This difference was not studied intensively because the agent was heat

labile.

Nakamura and Hartman (31) feel that dissimilarities between the behaviour of microorganisms in pure state and in mixtures may be a function of the presence of certain external conditions.

Williams, et al (56) found that high saliva counts of lactobacilli and low counts of yeast were most likely to be found when enterococci were present; this was in no way indicative of the presence or absence of carious lesions in the teeth.

Jugde and Pelczar (14) have shown a nutritional symbiosis between species of Streptococcus, Micrococcus, Neisseria and Lactobacillus, but not in a continuous flow system.

Parker and Snyder (38, 39) studied interactions between oral microorganisms in mixtures in their modification of the chemostat and the continuous flow system. Cultures of S. salivarius, S. aureus and V. alcalescens were used. Their results showed that S. aureus was inhibited by V. alcalescens, in the same system V. alcalescens had very little effect upon S. aureus. Further, while S. aureus had an increased generation time when growing with S. salivarius, the reverse was not true but the combination of V. alcalescens and S. salivarius showed mutual restriction of growth.

More recently, Scherp (48) has suggested that the role of a number of factors related to the etiology of caries and periodontal infections remains to be ascertained, and this is particularly true for the concept of a multiple rather than single etiologic agent for dental caries. However, new techniques will have to be developed to measure the effects of one organism on another to provide solutions to heretofore unanswerable problems on oral ecology. The application of a two-stage continuous culture system which allows study of two or more organisms growing singly

and in mixture offers an approach toward understanding the function of the microcosmos of the oral cavity. At the present stage of development of this apparatus, which is a modification of the chemostat, we feel it is important to establish the nature and degree of interactions between selected oral organisms, specifically species of the genera Streptococcus, Neisseria and Lactobacillus. The organisms are maintained in this system in the exponential phase at the maximum growth rate characteristic of the limiting growth factor and the flow rate applied. The usual changes of the physiological state of the population which are the normal consequences of growth in a closed system are thus avoided here. Because of the highly sensitive system we have used, an interaction, even a weak one, will be apparent. With sufficient replication the data should serve as a base-line for more refined and exact studies, as well as the source for biochemical analysis in attempt to explain the interactions.

MATERIALS AND METHODS

Organisms used in this study were the following: S. faecalis ATCC 8043, L. acidophilus ATCC 4357 and S. aureus, originally a departmental stock culture isolated from the oral cavity. These organisms were transferred once a month by stab inoculation in a yeast-brain heart infusion agar deep. S. aureus also was transferred once a month on a nutrient agar slant.

The cultures were transferred to an activating substrate at 24 hour intervals for at least four days before a test run was performed.

Culture media. The medium used for activation of L. acidophilus contained 1 per cent trypticase, 0.5 per cent yeast extract and 0.1 per cent sucrose. For S. faecalis 0.1 per cent trypticase and 0.1 per cent sucrose. For S. aureus a broth containing 0.1 per cent trypticase and 0.01 per cent thiamine hydrochloride was used for activation. For the purposes of growth and counting of bacteria in our system, two objectives were sought: (1) to find a plate counting medium which would be selective and still yield maximum counts of the selected organism: (2) to develop a chemostatic medium for each of these organisms.

Counting media. After numerous trials in order to get information enough for comparison it was found that Rogosa's SL agar adjusted to pH 5.5 gave the best counts for L. acidophilus. Although S. faecalis will grow on this agar in small quantities, no difficulties were found in differentiating between the two organisms on this medium; S. faecalis forms circular, white, smooth, convex colonies with a diameter of 1 mm,

L. acidophilus gives rough, brownish, flat colonies 2-3 mm in diameter.

S. faecalis was found to give maximum counts on brain-heart infusion agar with 6.5 per cent sodium chloride added whereas S. faecalis shows circular, convex, white colonies with a diameter of 1 mm. S. aureus also will grow on this medium to a certain extent but will give bright yellow colonies, which were especially pronounced if the plates were kept at room temperature or in the cold room for 3-4 hours. S. aureus will give maximum counts on a glycine-tellurite agar suggested by Zebovitz, Niven and Evans (61). The components of this medium are listed in appendix 1. This medium will support growth of S. aureus showing circular, black colonies after 24 hours incubation at 36° C. and the medium is selective for this organism.

Chemostatic media. The second part of the preliminary studies consisted of elaborating a chemostatic medium for each bacterium.

For S. faecalis 0.1per cent trypticase and limiting substance 0.01 per cent sucrose was used. For S. aureus 0.15 per cent potassium dihydrophosphate, 0.35 per cent dipotassium phosphate, 0.01 per cent magnesium sulphate, 0.5 per cent vitamin-free casamino acids and limiting substance 0.1 mg per liter was used, and for L. acidophilus 0.1 per cent trypticase, 0.1 per cent yeast extract and limiting substance 0.01 per cent sucrose was used. Figures 1, 2 and 3 show the relation between growth measured as optical density and time for each of the three bacteria in their chemostatic medium.

Appendix 2 gives a summary of the substrates used for activation, chemostatic growth and selective counting for the studied microorganisms.

Closed system studies. Before the study of these three organisms in the continuous flow system for possible interactions it was necessary

to determine the generation time for each organism in the medium furnished the final mixture of organisms in the mixed culture cell. Here each organism was grown in 120 ml of medium freshly made and sterilized in a 150 ml milk dilution bottle. The pH of this medium was adjusted to pH 7.0 except for L. acidophilus where it was pH 6.8. The stirring effect was produced by a teflon coated magnet $\frac{1}{2}$ inch long turning at a speed regulated with a magnestir motor so that no vortexing was present. This homogeneous mixture appeared to be identical with mixing used in the final mixed culture system.

The culture medium was contained in 150 ml pyrex milk dilution bottles. Immediately after inoculation the bottle was incubated in a glycol-water bath, the temperature of which was kept constant at 37° C. by means of a thermostatically regulated pump constructed by Radiometer, Copenhagen. Glycol was added in order to minimize water evaporation. At the times indicated on Figures 8, 9, 12, 13, 16 and 17, a 1.0 ml sample was pipetted from the culture bottle and diluted in 99 ml sterile diluation blanks of 0.1 per cent peptone water buffered at pH 7.0 with 0.067 M phosphate buffer as follows: 10^2, 10^4 and 10^6. Whenever L. acidophilus was studied the first dilution bottle contained 10-20 glass beads; these were found to break the chains of cells when shaken, so that each single colony originated from a single bacterial cell. The diluted culture sample was plated out in dilutions of 10^3, 10^4, 10^5, 10^6 or 10^7 according to the phase of growth of this organism on the selective medium for counting of that particular organism. Streak plates were made by means of sterile bent glass rods. The glass rods were wrapped in separate packages of aluminum foil and sterilized to secure streaking under aseptic conditions in as high a degree as practically possible.

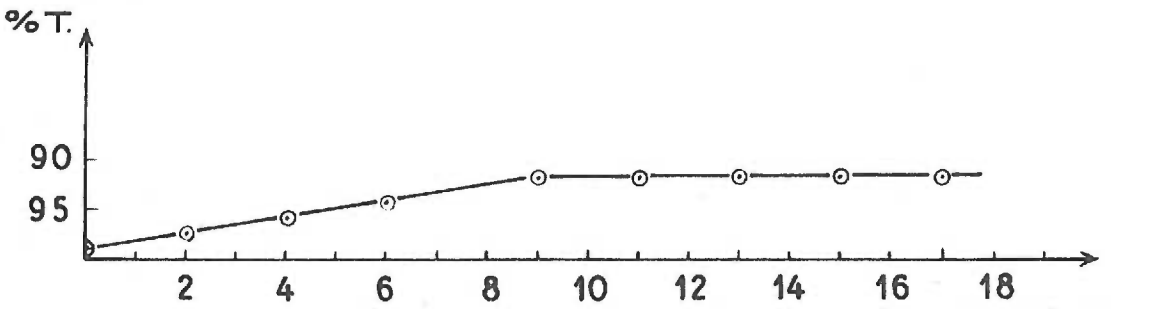


Fig.1. Relation between growth measured as optical density at 560 mμ and time for Streptococcus faecalis in the chemostatic medium for this microorganism.

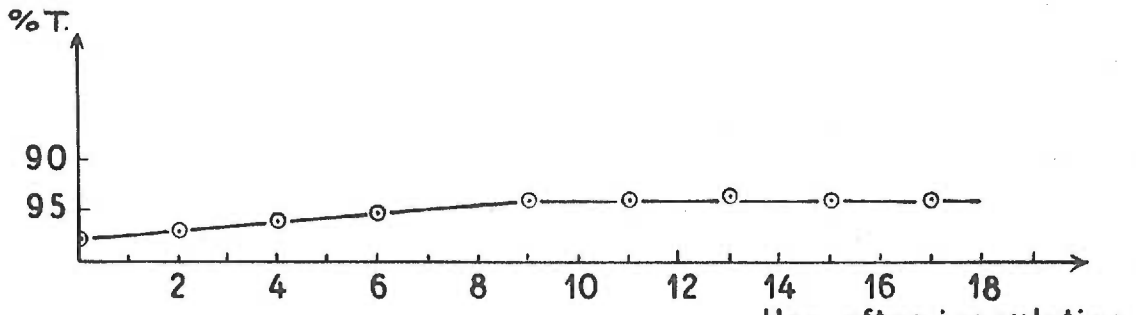


Fig.2. Relation between growth measured as optical density at 560 mμ and time for Lactobacillus acidophilus in the chemostatic medium for this microorganism.

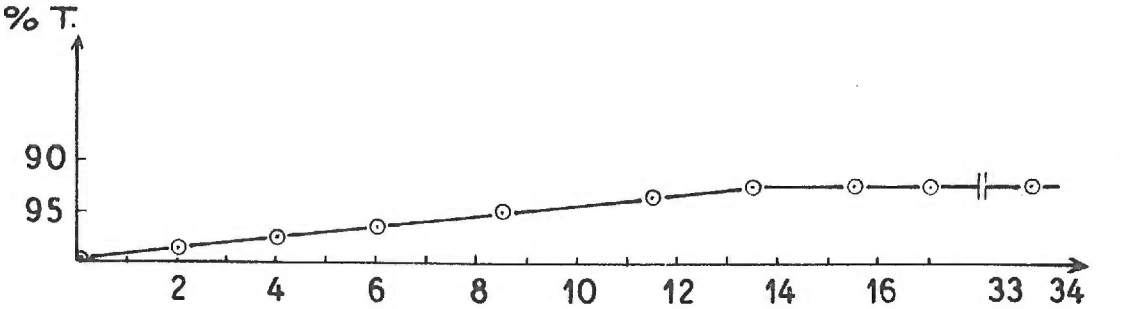


Fig.3. Relation between growth measured as optical density at 560 mμ and time for Staphylococcus aureus in the chemostatic medium for this microorganism.

The plates were incubated at 36° C. 24 hours for S. aureus; 48 hours for S. faecalis and five days for L. acidophilus. Readings of optical density of 4 ml of the culture sample were recorded in a spectrophotometer ("Spectronic 20") at 560 mu immediately after sampling to correlate optical density (O.D.) with population. Also the pH of the sample was determined by pHydrion paper. Counts of colonies originating from viable bacterial cells were then made with an electrical colony counter. Counts of about 100 colonies per plate were considered preferable; by experience these gave the greatest accuracy. Each sampling was done in duplicate and all streakings in replicates of two.

The number of colonies present related to the time of sampling were plotted on log paper showing the growth curve for each single bacterium in the medium used. The generation time in the logarithmic phase of growth was computed from following formula:

$$\theta g = \frac{\log X_2 - \log X_1}{t \log 2}$$

where θg equals generation time, X_2 the viable cells present at time of sampling, X_1 viable cells present at the previous sampling and t equals the time interval between the two samples. When the generation time is known, k illustrating a growth characteristic for that particular organism can be computed from:

$$K = \frac{0.694}{\theta g}$$

Following this the critical total flow F in the final continuous flow system to the mixed culture cell in order to maintain maximum growth for that organism can be determined. F/V must exceed $k_o m$, the maximum growth rate.

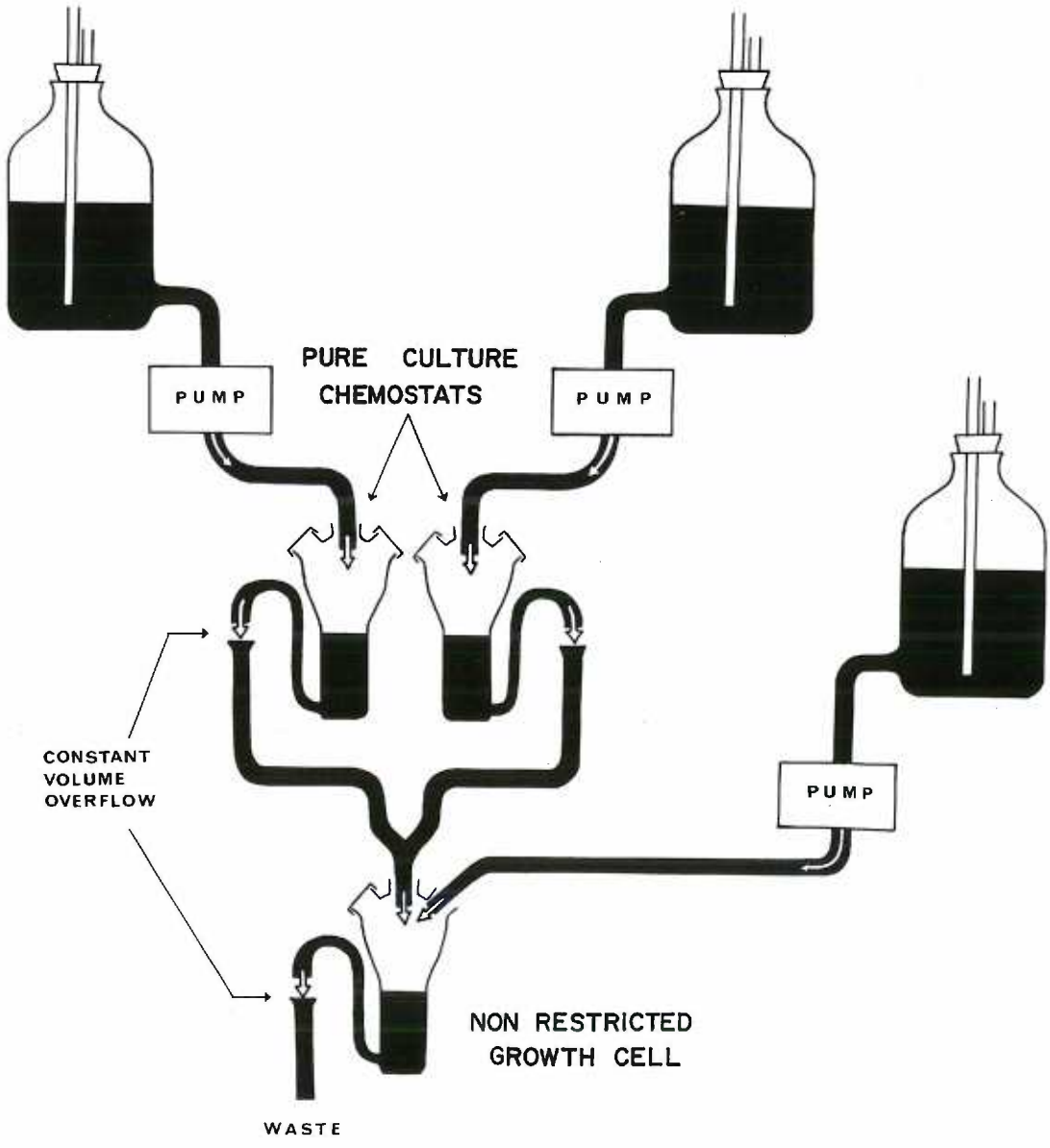
Continuous flow studies.

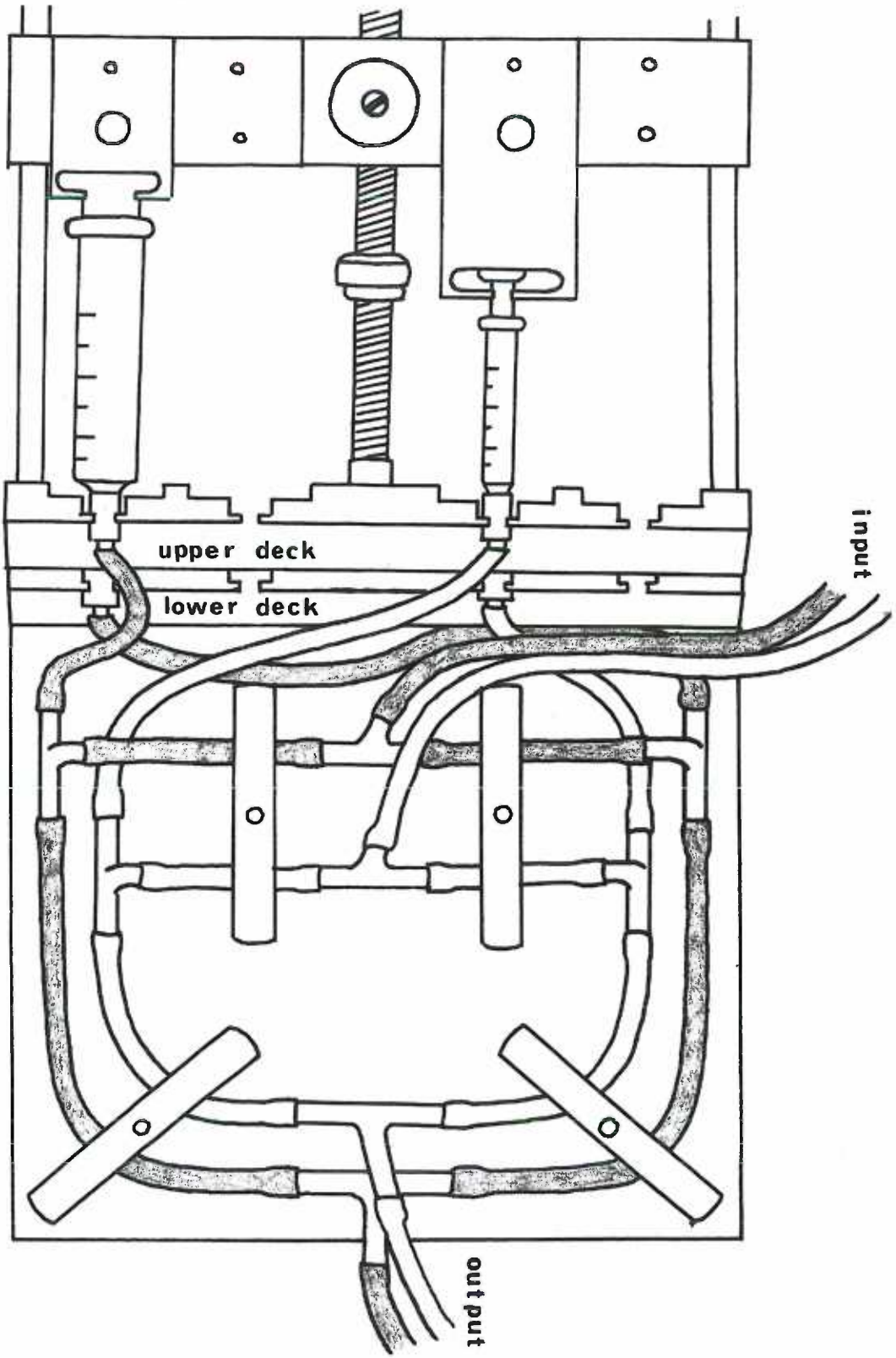
Apparatus. The culture system used in these studies was considerably modified from the original system employed in this laboratory for defined mixed growth (38).

A schematic flow chart of a two culture unit is given in figure 4. About 1.5 liters of medium for each chemostat was stored in Mariotte flasks and supplied to the culture cells through a variable speed infusion pump (Harvard Apparatus Company). This pump has two decks each holding a maximum of 4 syringes. Figure 5 shows the principle of the pump. The upper deck is pushed forward by a screw gear for output of the pump, while the syringes on the lower deck are filled by moving the rack in opposite direction. Limit switches determine the length of travel of each deck and automatically reverse the direction of motion. At the time of reversal the upper syringes are filled and the lower unit is used for output. In this way a continuous flow of fluid is maintained. Direction of flow in the rubber tubing is determined by two pairs of solenoids operated pinch clamps which are activated by its limit microswitches. Thus, with the upper deck on output and the lower deck on input, fluid will flow from the Mariotte flask to the junction between the upper solenoids (on figure 5) and as the upper left-hand solenoid and the lower right-hand solenoid are closed, flow only to the lower deck syringe which is being retracted by the screw gear. At the same time the filled syringe on the upper deck is being pushed forward by the action of a contri rotating screw, and since fluid can not go through the closed upper left-hand solenoid it must flow through the open lower left-hand side of the loop to output to the growth cell.

CONTINUOUS FLOW

MIXED CULTURE SYSTEM




upper deck
lower deck
input
output

Tubing used was intravenous tubing 1/8 by 1/32 amber with inside walls treated to prevent them from sticking when autoclaved or pinched off. The entire system of loops could be removed from clips and sterilized by autoclaving.

An outline drawing of the chemostat is shown in figure 6. These chambers were made of Pyrex glass with standard taper joints to permit interchangeability of drip points. The lower portion was cylindrical and 30 mm in diameter with a syphon overflow to maintain a constant volume of 83 ml.

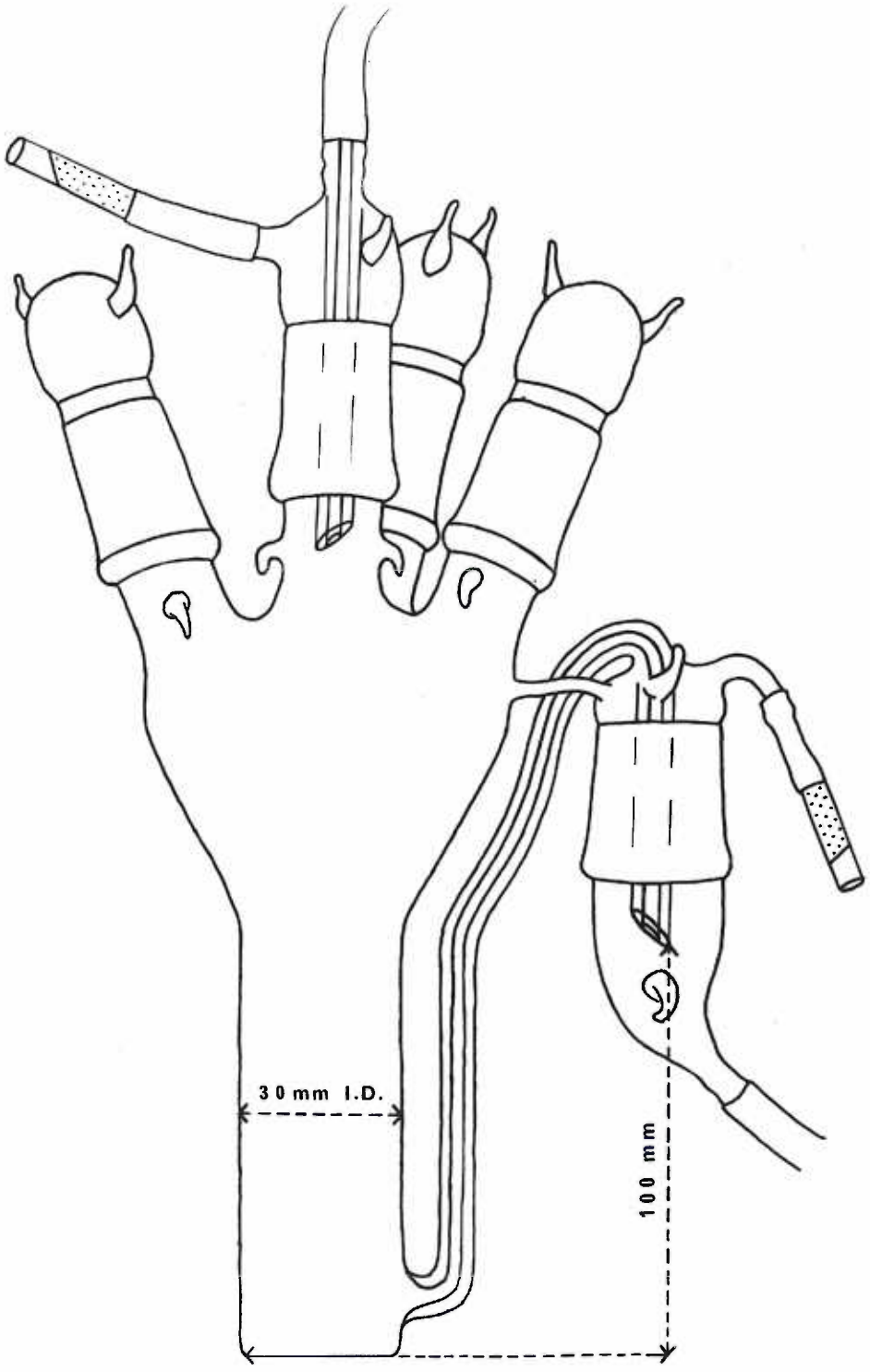
Contents of the chemostats were stirred by a $\frac{1}{2}$ x $\frac{1}{4}$" teflon coated magnet activated by a Magnestir motor placed below a plexiglass water bath containing the chemostats.

Temperature in the water bath was maintained at 37° C. by circulating a glycol-water mixture from a thermostatically regulated reservoir (Radiometer, Copenhagen).

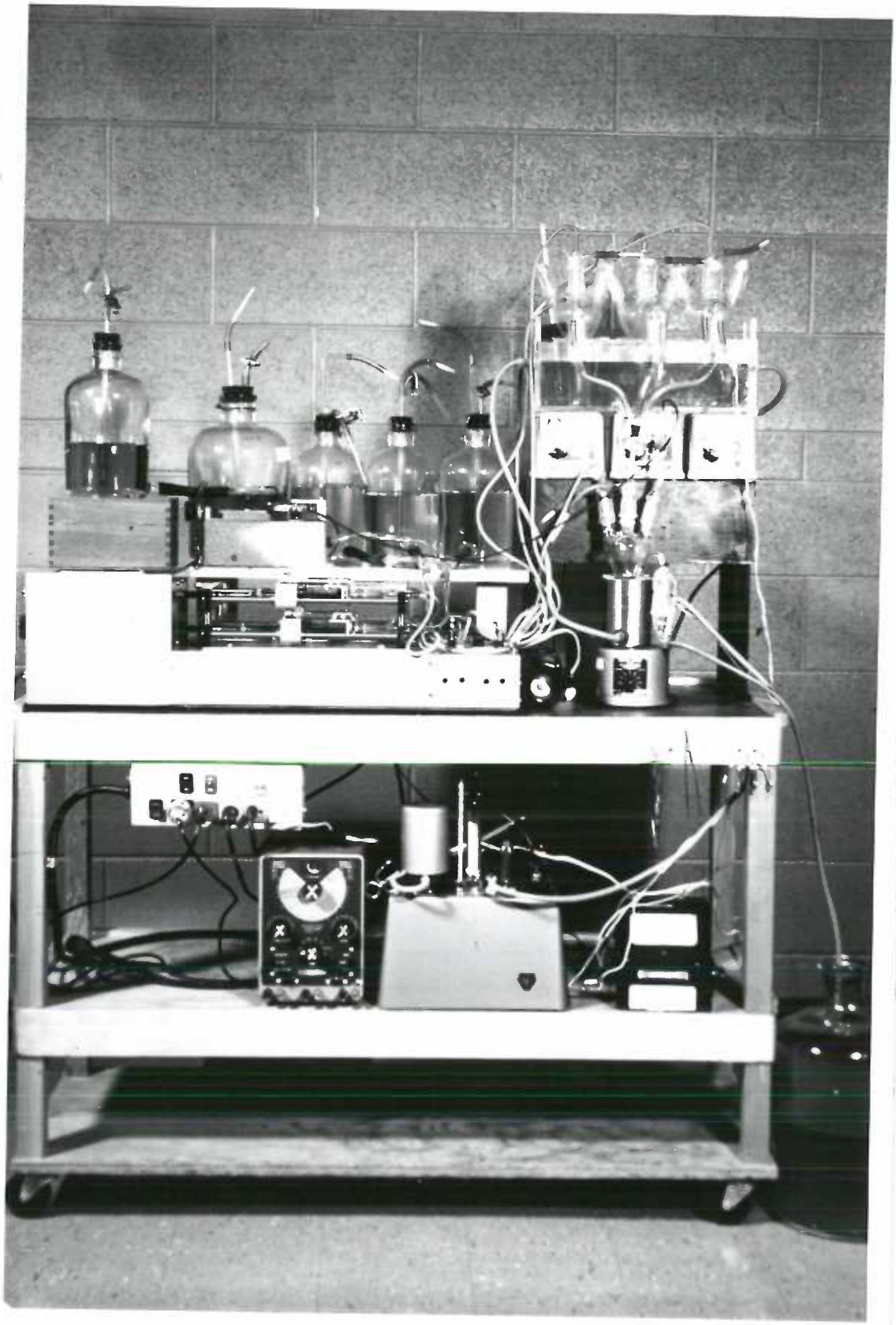
The growth cells were connected with a Y glass joint for a two cell unit and a tri-armed joint connected the chemostats with the mixed culture cell where combination of three organisms were studied. Figure 7 shows the apparatus and set-up for study of combination of three organisms.

When the combination of these three organism was studied, additional flow was found necessary in order to provide more fresh medium to the mixed culture cell than could be delivered by the infusion pump. Therefore, medium was led from a 2 liters flask via a peristaltic pump to a drip joint at the periphery of the mixed culture cell.

Preparation and sterilization of the apparatus. Each single unit



CONTINUOUS CULTURE CELL



consisting of reservoir flask, two syringes, chemostat and connecting rubber tubing was prepared and sterilized separately.

Prior to sterilization the syringes were washed several times in water, cleaned with acetone, dried and coated with a thin film of Beckman's Desicote, the excess of which was removed with acetone and a thin layer of white petroleum jelly applied. The excess of petroleum jelly was removed with paper towels.

The medium was made and 1.5 liters placed into each of the Mariotte flasks belonging to the chemostats and 3 liters in the reservoir connected to the mixed culture cell. Chemostats and mixed culture cell was charged with 80 ml medium. The syringes of the pump were partly filled with the medium, and were connected to the chemostats and the mixed culture cell. The rubber stopper for each Mariotte flask was lubricated with silicone lubricant and a brass wire was twisted around the neck of the bottle and over the cork to hold it in position. The connection between the medium flask and the rubber tubing from the Mariotte flask was pinched off with a hemostat and the short glass tube in the Mariotte flask left open to the air to prevent boiling of the medium. All joint surfaces on the chemostat as well as the inside of the joint were lubricated with a thin film of the silicone lubricant.

Because standard taper joints were used they could be covered with paper or aluminum foil, autoclaved separately and then covered aseptically.

For sterilization each glass cell was placed in a steel wire basket and held in upright position by cotton towels to avoid overflow. These were autoclaved for 30 minutes at 121° C. and cooled slowly to avoid boiling out the medium.

Procedure of continuous flow studies of mixed cultures. A heavy broth culture of each organism used in the experiment was prepared and incubated for 24 hours except when L. acidophilus was studied, a 36 hours culture was then used. Two ml of this culture was inoculated into the proper chemostat and the circulating water bath and the magnestir motor was started. After 12 to 15 hours the flow to the chemostats was started. In the first part of the study a slow flow rate of 4.7 ml per hour was used resulting in a retention time of about 16 hours in each chemostat.

Flow was maintained for 3 hours in order to let the organisms grow in the growth cell achieving a steady state for the bacteria. Before the flow of nutrients was started to the mixed culture cell a sample of 5 ml was pipetted out of each chemostat and 1 ml of this sample was diluted in 10^2, 10^4 and 10^6 and spread on agar selective for that particular organism in dilutions 10^3, 10^4, 10^5 and 10^6 for S. faecalis and S. aureus, when L. acidophilus was included the first dilution bottle contained 10-20 glass beads to be shaken with the sample and thus breaking up the chains of organisms. For this organism, plating was done in 10^2 dilution. The diluent employed was 0.1 per cent peptone in water adjusted to pH 7.0 with addition of phosphate buffer. Streaking was done with sterile bend glass rods. The plates then were incubated at 36° C. An additional 4 ml of the sample was used for recording the optical density to check a permanent steady state population in the chemostats. This reading was done in a spectrophotometer at 560 mu.

Samples were taken from the mixed culture cell 3 hours after flow

to the mixed population was started. The total flow was in this part of the study 22 ml per hour for pairs of organisms and 27 ml per hour when combination of three organisms were studied. This means that the bacteria under these conditions will exist with each other over extended periods of time, which means that action of physical factors and of metabolic products and competition for the provided nutrients upon the generation time will be more distinct than where the exchange of nutrients. In this part of the experiments the growth rate constant k was o.3 k_om for the bacterium in each pair that had the shortest generation time and correspondingly the largest k_om.

One ml was pipetted out from the mixed glass cell and diluted in diluent composed as the diluent for the samples from the chemostats. At the same pH was determined by means of pHydron paper.

Samples were taken at intervals of $\frac{1}{2}$ to 1 hour, all were done in replicates of two and further all plates of each replicate were made in duplicate. Nine replications of each system design were considered to give significant results at a 95 per cent level.

Plates were then incubated at 36° C. for a period, considered to give optimal counts for each single bacterium; a period of 24 hours was allowed for S. aureus, 48 hours for S. faecalis (in this case the plates were kept in the cold room for at least 3 hours before counting to get a more distinct difference in color between the colonies of S. faecalis and S. aureus). Five days incubation was necessary before L. acidophilus could be counted.

The second part of the experiment was then started at a speed corresponding to gear 5 of the infusion pump equal to approximately 90 ml per hour with the syringes used. After a sample had been taken from

the chemostats with dilution, plating and readings of the optical density to check the presence of the steady state and the maintenance of the population level in the chemostats the 10 ml syringes were exchanged with 30 ml syringes to provide the required flow to the mixed culture cell and at the same time maintain the same population level in the chemostats. The flow from the 2 ml syringes used for S. faecalis and S. aureus at this speed was equal to 11.8 ml per hour. From the 1 ml syringes used for L. acidophilus it was 4.7 ml per hour. For this organism it was found impossible to maintain a steady state if the 2 ml syringes were used; therefore, these were exchanged with 1 ml syringes. The above flow provides a retention time of about 6 hours for S. faecalis and S. aureus and about 16 hours for L. acidophilus. The total flow where pairs were studied would then be 90 ml per hour. Exchange of syringes could be done without contamination of the system.

Whenever combination of three organisms was studied the additional flow to the mixed cell would provide 10 drops in 34 seconds equal to 40 ml per hour. Thus, the total flow in this case would be 140 ml per hour, and providing a flow exceeding $k_o m$ V. Exceptional precision in determining the flow rate to the mixed cell was not required because total volume through the cell could be determined by simply measuring the output in a volumetric cylinder at the waste port.

For the studied pairs the flow in total was getting as close to this requirement as it was possible with the equipment available. The sampling, time intervals between samples, dilutions, plating and streaking technique here were as in the first part of the experiment.

Pairs studied were:

S. faecalis - S. aureus

S. faecalis - L. acidophilus

S. aureus - L. acidophilus

Combination of all three organisms was studied as the final experiment.

Results were expressed as generation time for each bacterium in the mixed growth cell as the most exact expression for possible interaction and also as a replicable expression.

The mathematical expression for generation time was:

$$\theta g = \frac{\log X_2 - \log X_1}{t \log 2}$$

where in this instance X_2 equals the volume of the culture in the mixed culture cell multiplied with the amount of cells per ml added to the amount of cells drained out during the time unit from this is substrated the amount of cells present in the mixed culture at the time of the previous sampling. X_1 equals the amount fed into the mixture per hour from the chemostat where that particular organism is growing. t equals the time unit - here 60 minutes.

Results are reported for each single organism compared with the generation time found for the same organism when growing in pure culture in the medium provided to the mixed culture in a closed system under conditions as close to those for the continuous flow system as possible.

FINDINGS

For pairs of organisms. The results show that in the combination of S. faecalis with S. aureus, the former is stimulated significantly (figure 8 and table 1, figure 11 and table 2) both at $k_o m$ and at 0.3 $k_o m$. It has a generation time of 10 minutes compared to 40 minutes in the same medium in pure culture. Also the results seem to indicate a stronger stimulation in the part of the experiment where a slow flow of nutrients was used, possibly because the additional hold-up of S. aureus may allow for accumulation of beneficial products. S. aureus in the same system at a flow of $k_o m$ V shows a varying generation time with extremes of 66.9 to 13.7 minutes. The cause of this variation was not known but appeared to be related to mechanical problems in the procedure of sampling and mixing. At 0.3 $k_o m$ the staphylococcus is strongly inhibited with a generation time longer than the retention time in the growth cell, which is about 4 hours. In pure culture this organisms had a generation time of 36 minutes (figure 9, table 1 and figure 10 and table 3).

Paired mixtures of S. aureus and L. acidophilus display a very unusual interaction. S. aureus at $k_o m$ apparently has a very short generation time of 11.6 - 5 minutes (figure 15 and table 5), with the lowest values in the last part of the study. Again this seems to indicate that some length of time is necessary for action in the mixed population. At 0.3 $k_o m$ the generation time is closer to that of the pure culture - 36 minutes (figure 12 and table 4) in the same sub-

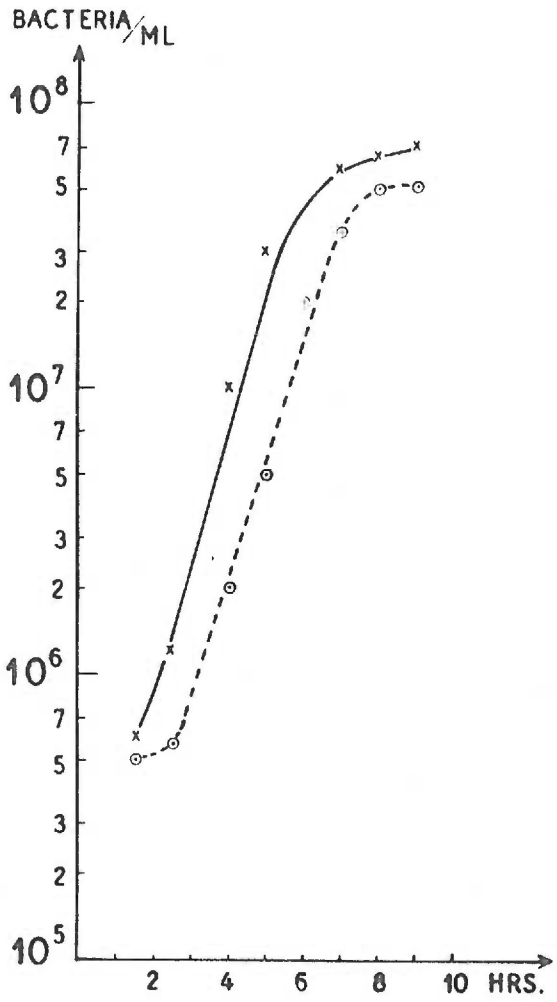


Fig. 8. S. faecalis in closed system. Nutrient: medium for pair of S. faecalis and S. aureus.

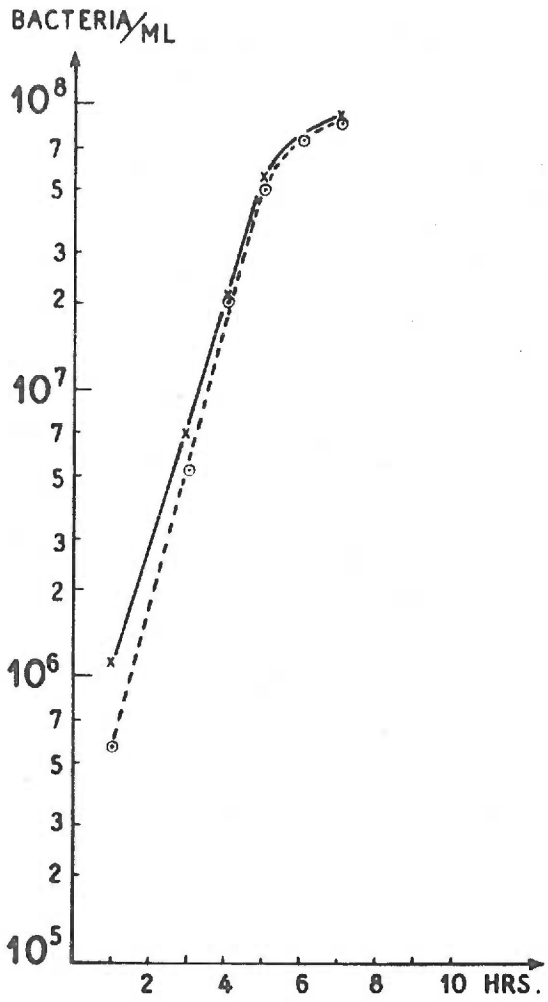


Fig. 9. S. aureus in closed system. Nutrient: medium for pair of S. faecalis and S aureus.

	Θg	$k_o m = \frac{\ln 2}{\Theta g}$	F >
STREPTOCOCCUS FAECALIS	40.0 MIN.	1.04	86.3 ml/HR.
STAPHYLOCOCCUS AUREUS	36.4 MIN.	1.17	97.1 ml/HR.

Table 1. Values of Θg, $k_o m$ and total flow for S. faecalis and S. aureus, each in pure culture in closed system with the medium for the combination of these organisms.

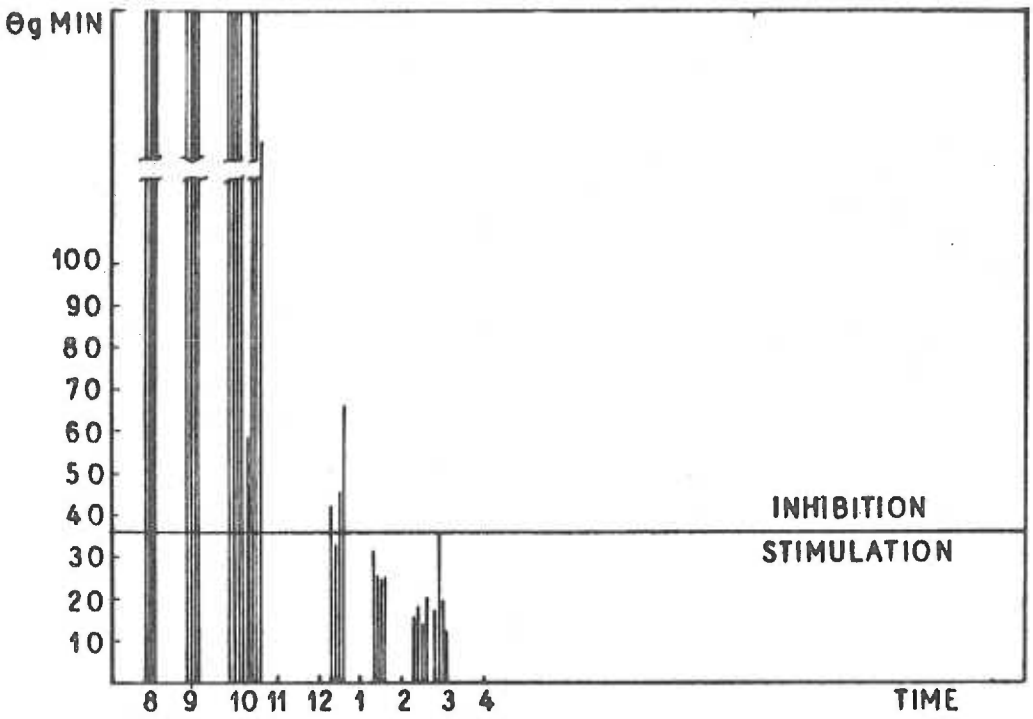


Fig.10. FLOW RATE=0.3k_0m FLOW RATE=k_0m
Generation times of S. aureus in mixed continuous flow culture system with S. faecalis.

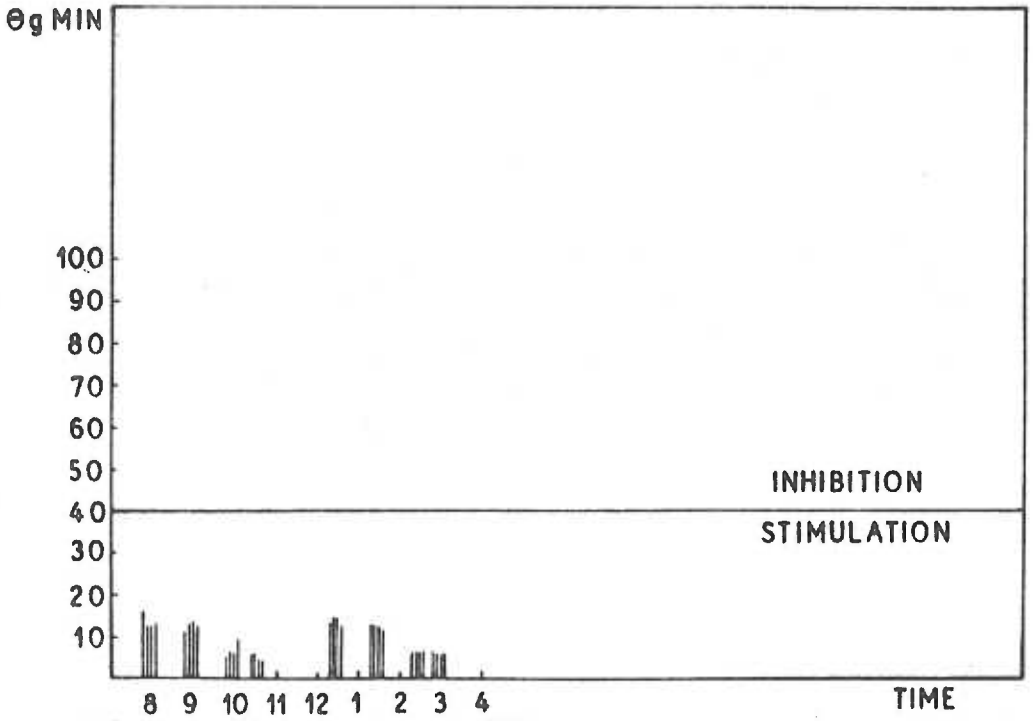


Fig.11. FLOW RATE=0.3k_0m FLOW RATE=k_0m
Generation times of S. faecalis in mixed continuous flow culture system with S. aureus.

TIME	0.3 $k_o m$				TIME	$k_o m$			
	A	A_1	B	B_1		A	A_1	B	B_1
5:30	16.3	12.5	13.3	14.3	12:30	14.0	14.7	14.7	14.0
7:00	12.6	13.7	13.9	13.1	1:30	12.7	12.5	12.6	12.9
7:30	5.9	7.3	6.8	9.7	2:30	6.2	6.5	5.9	6.7
8:00	5.8	6.1	5.3	5.5	3:30	6.4	6.1	7.6	5.9
In closed system S. faecalis: $\Theta g = 40$ min.									

Table 2. GENERATION TIMES OF S. FAECALIS IN MIXED CONTINUOUS CULTURE WITH S. AUREUS.

TIME	0.3 $k_o m$				TIME	$k_o m$			
	A	A_1	B	B_1		A	A_1	B	B_1
5:30	∞	∞	∞	∞	12:30	42	34.1	46.3	66.9
7:00	∞	∞	∞	∞	1:30	31.7	26.2	25.1	25.4
7:30	∞	∞	∞	∞	2:30	15.6	18.8	14.8	20.1
8:00	60.2	∞	∞	129 min	3:30	18.4	27.3	19.6	13.7
In closed system S. aureus: $\Theta g = 36.4$ min.									

Table 3. GENERATION TIMES OF S. AUREUS IN MIXED CONTINUOUS CULTURE WITH S. FAECALIS.

strate, possibly because of the slow flow of nutrients, which does not allow the stimulating action of L. acidophilus upon S. aureus to exceed the action of competition for nutrients. L. acidophilus is in this pair stimulated at $k_o m$ with a generation time of 24 - 62.3 minutes (figure 14 and table 6) compared to 97.5 minutes in pure culture (figure 13 and table 4). When the flow rate is reduced to 0.3 $k_o m \times V$, the generation time at first is longer than the retention time in the mixed culture cell. However, two hours after sampling was started the organism became strongly stimulated with generation times of 27 to 15 minutes (figure 14 and table 6). This might be caused by the action of S. aureus on L. acidophilus accumulating at the lower flow rate.

The combination of S. faecalis and L. acidophilus results in a stimulation of S. faecalis (figure 18 and table 8) with generation times of 10 - 18 minutes compared with generation time of 37.3 minutes (figure 16 and table 7) in pure culture, while L. acidophilus (figure 19 and table 9) has a generation time longer than the retention time in the growth cell. These results are valid at both flow rates of $k_o m \times V$ and 0.3 $k_o m \times V$. In the pure culture experiments with a medium similar to that of the continuous flow for the mixed culture system L. acidophilus had a generation time of 107 minutes (figure 17 and table 7).

For the triple combination. The simultaneous inclusion of all three species in mixture shows a stimulation of S. faecalis (figure 23 and table 11) both at $k_o m$ with generation times of 22 to 9 minutes and

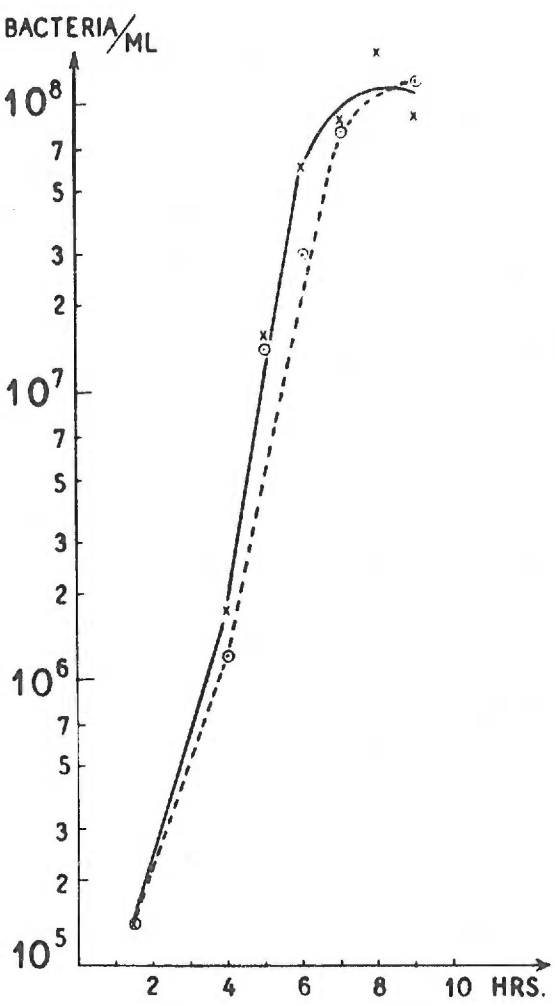


Fig. 12. S aureus in closed system. Nutrient: medium for pair of S. aureus and L acidophilus.

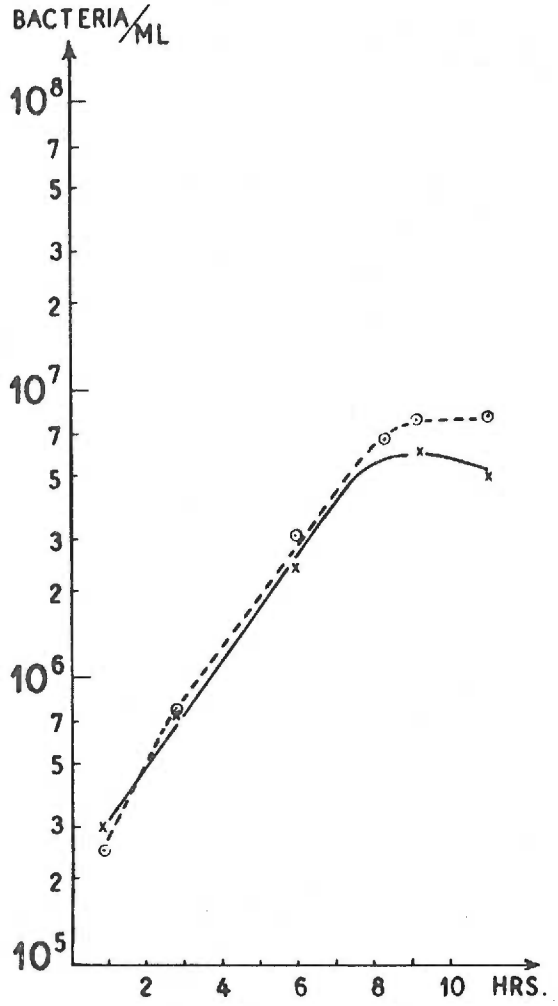


Fig. 13. L. acidophilus in closed system. Nutrient: medium for pair of S aureus and L. acidophilus.

	Θg	$k_0 m = \frac{\ln 2}{\Theta g}$	F >
STAPHYLOCOCCUS AUREUS	36.0 MIN.	1.16	96.28 ml/HR
LACTOBACILLUS ACIDOPHILUS	97.5 MIN.	0.42	34.86 ml/HR

Table 4. Values of Θg, $k_0 m$ and total flow for S. aureus and L. acidophilus, each in pure culture in closed system with the medium for the combination of these organisms.

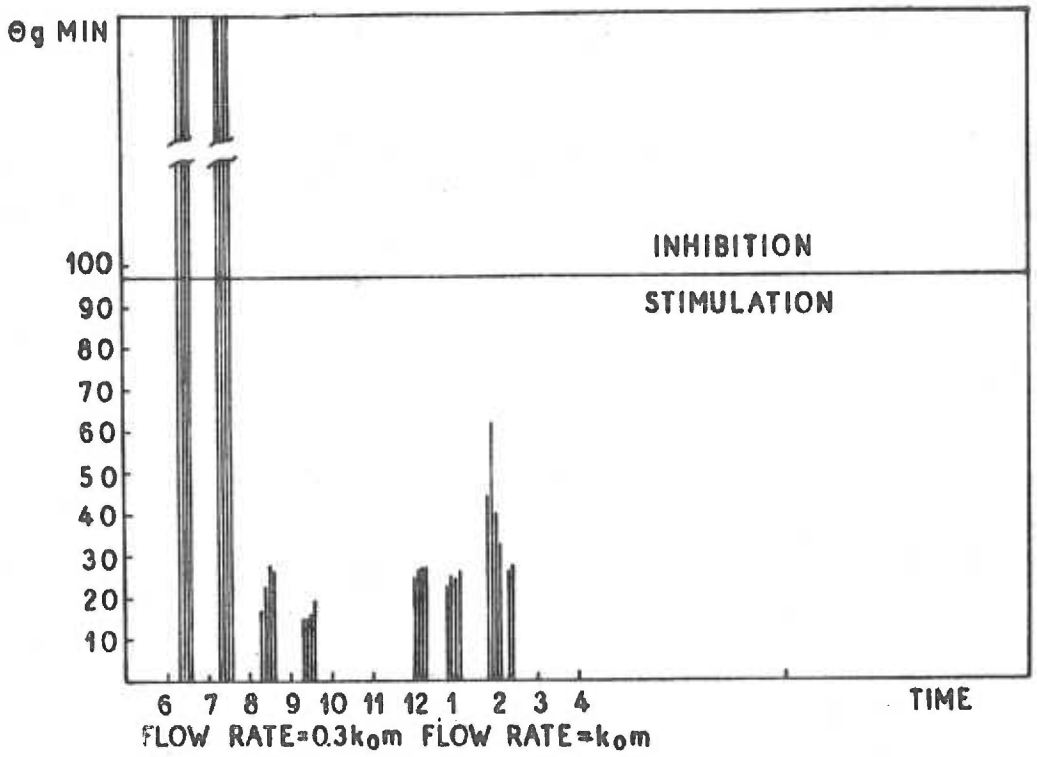


Fig.14. Generation times of L.acidophilus in mixed continuous flow culture system with S. aureus

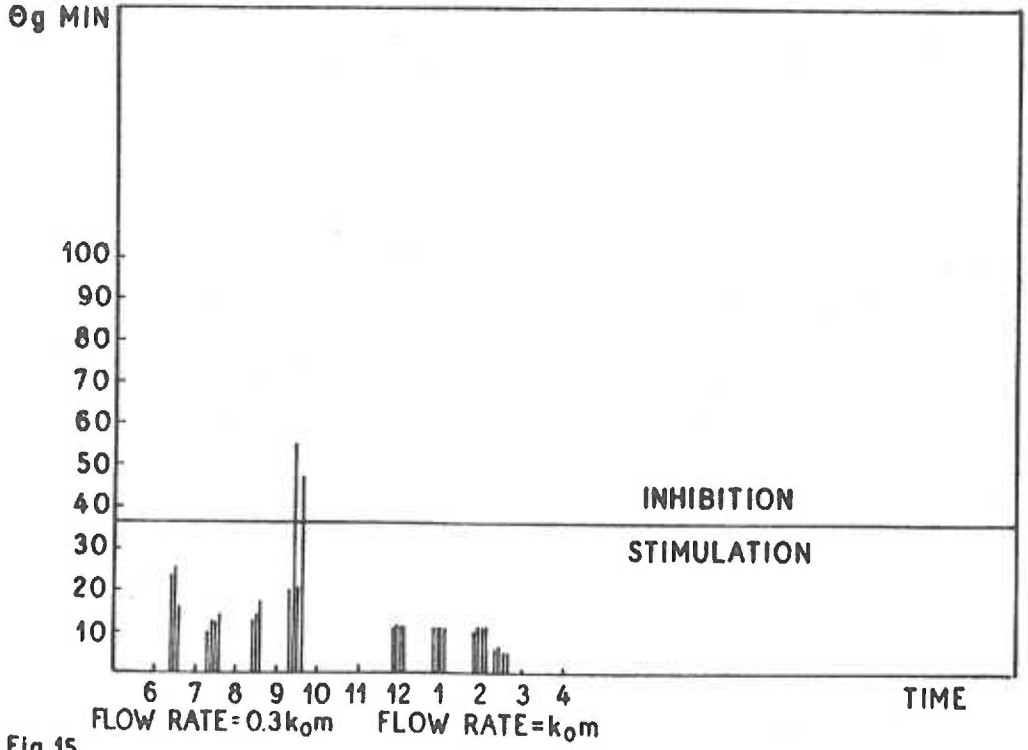


Fig.15 Generation times of S. aureus in mixed continuous flow culture system with L acidophilus.

TIME	0.3 k_om				TIME	k_om			
	A	A_1	B	B_1		A	A_1	B	B_1
6:30	∞	24.4	24.4	16.4	12	10.9	11.1	10.6	10.9
7:30	10.3	13.0	13.1	13.6	1	11.1	10.8	11.6	10.7
8:30	∞	13.4	14.2	16.9	2	10.3	10.6	10.3	10.7
9:30	20.5	54.7	19.6	36.9	2:30	5	5.2	5	5.1
In closed system S. aureus: $\Theta g = 36$ min.									

Table 5. GENERATION TIMES OF S. AUREUS IN MIXED CONTINUOUS CULTURE WITH L. ACIDOPHILUS.

TIME	0.3 k_om				TIME	k_om			
	A	A_1	B	B_1		A	A_1	B	B_1
6:30	∞	∞	∞	∞	12	23.8	26.6	26.9	27.0
7:30	∞	∞	∞	∞	1	23.2	24.4	22.9	25.8
8:30	17.7	22.3	27.0	25.1	2	34.1	62.3	40.1	31.7
9:30	14.8	14.7	15.7	18.4	2:30	25.8	26.6		
In closed system L. acidophilus: $\Theta g = 97.5$ min.									

Table 6. GENERATION TIMES OF L. ACIDOPHILUS IN MIXED CONTINUOUS CULTURE WITH S. AUREUS.

at 0.3 $k_o m$ where generation times of 11.3 to 8.2 minutes were obtained. Generation time of this species in pure culture is 25.5 minutes (figure 20 and table 10). When a flow rate exceeding 0.3 $k_o m \times V$ is used, the stimulation is not very strong but at the same time the variation within the generation time is very small. This might be interpreted as approximating a steady state.

S. aureus also is stimulated (figure 24 and table 12), compared to generation time in pure culture of 39 minutes (figure 21 and table 10). For this organism it is also true that the strongest action and at the same time the smallest variation is at 0.3 $k_o m$ where generation time equals 19 - 14.8 minutes, when for $k_o m$ the generation time is 18.8 - 31 minutes.

L. acidophilus was strongly inhibited (figure 25 and table 13) compared to that in the pure culture of 76 minutes (figure 22, table 10). When the slow rate was as close to 0.3 $k_o m \times V$ as possible, only one generation time could be computed indicating a generation time of 86 hours. In all other instances in this mixture, generation times were computed as infinity. At $k_o m$ the generation times varied from values close to that of the pure culture 69.5 - 78.5 minutes to values showing stimulation 40 - 27 minutes. The overall result in this part of the experiments seems to indicate a stimulation.

Results in respect to reliability of system and apparatus. The set-up of the equipment worked satisfactorily. However, during the study it was found desirable to be able to use several different combinations of flow rates which to some extent could be done, as we did, by different sizes syringes. This was especially necessary when a slow growing organism such as L. acidophilus was studied. It would be of great

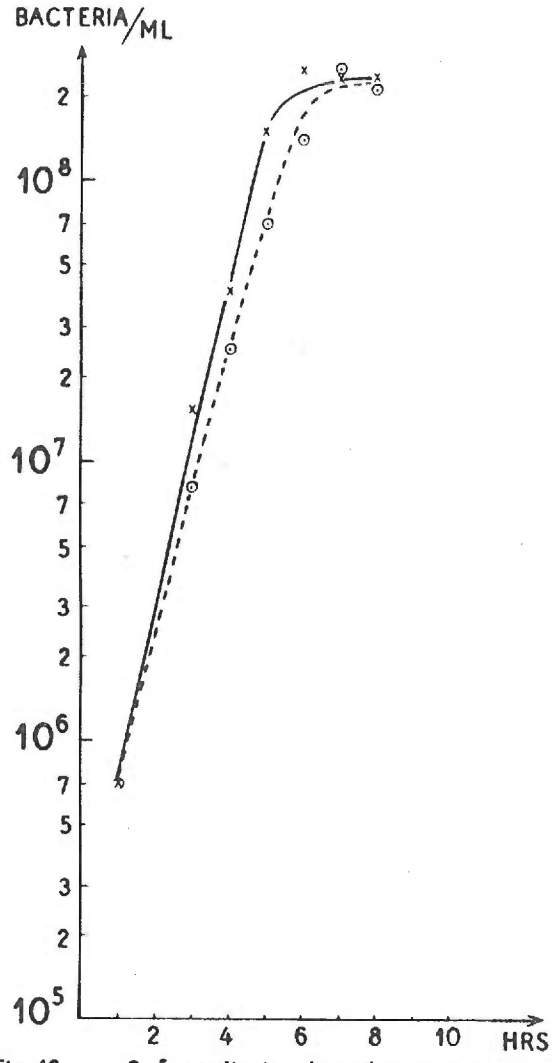


Fig. 16. S. faecalis in closed system. Nutrient: medium for pair of S. faecalis and L. acidophilus.

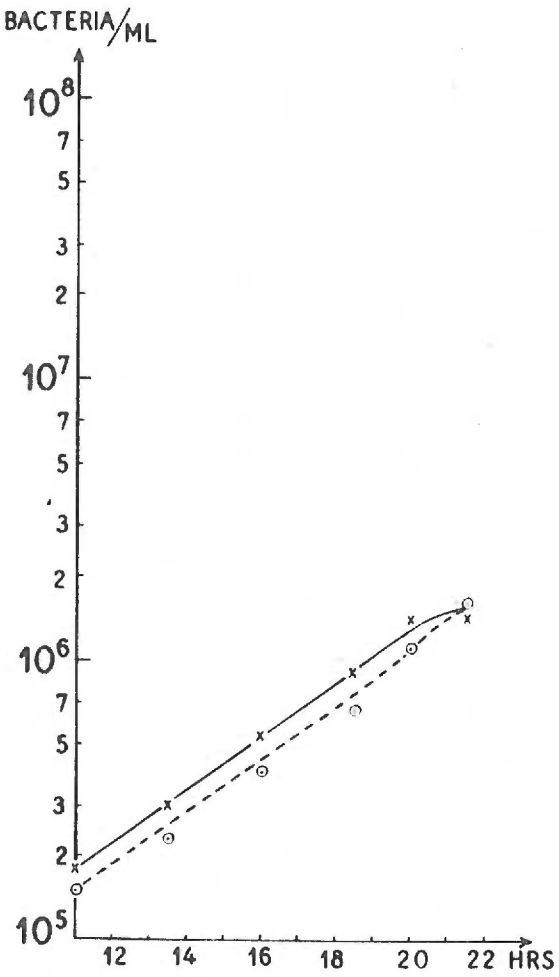


Fig. 17. L. acidophilus in closed system. Nutrient: medium for pair of S. faecalis and L. acidophilus.

	Θg	$k_o m = \frac{\ln 2}{\Theta g}$	F >
STREPTOCOCCUS FAECALIS	37.3 MIN.	1.12	92.96 ml/HR
LACTOBACILLUS ACIDOPHILUS	107.0 MIN.	0.39	29.37 ml/HR

Table 7. Values of Θg, $k_o m$ and total flow for S. faecalis and L. acidophilus, each in pure culture in closed system with the medium for the combination of these organisms.

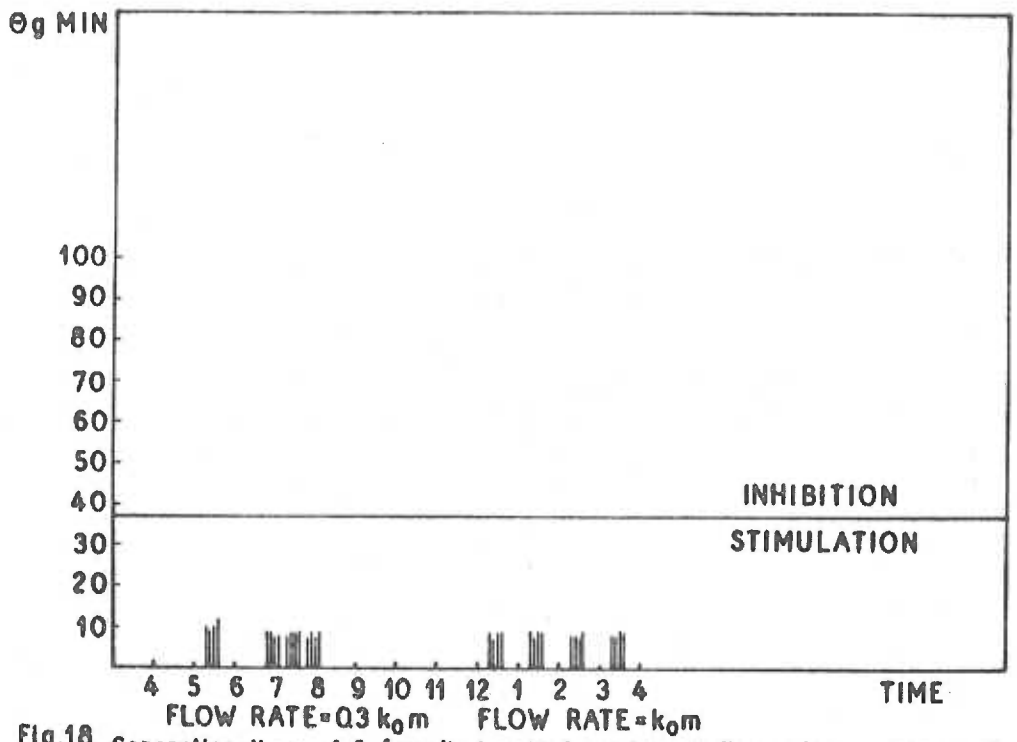


Fig.18. Generation times of S. faecalis in mixed continuous flow culture system with L. acidophilus.

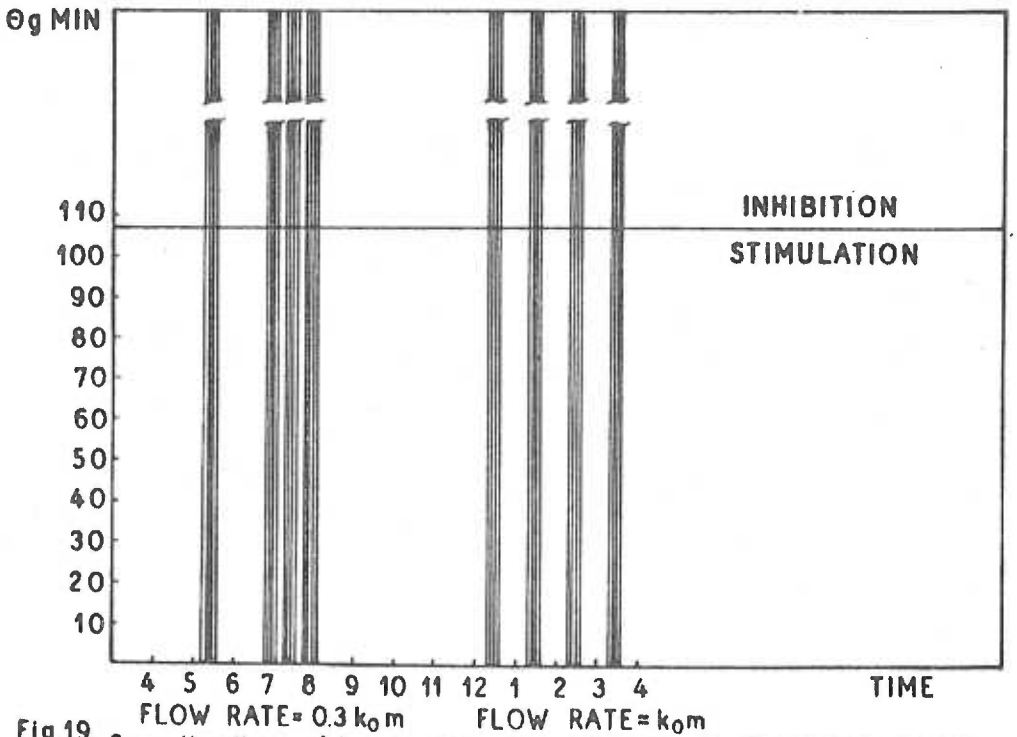


Fig.19. Generation times of L. acidophilus in mixed continuous flow culture system with S. faecalis.

TIME	0.3 k_0m				TIME	k_0m			
	A	A_1	B	B_1		A	A_1	B	B_1
5:30	9.7	9.2	9.9	11.9	12:30	8.6	8.2	8.6	8.7
7:00	8.6	8.8	8.3	8.1	1:30	8.7	8.4	8.2	8.5
7:30	7.9	8.9	9.1	8.8	2:30	8.0	8.1	8.1	8.8
8:00	8.1	8.6	8.3	8.7	3:30	8.0	8.4	8.5	8.5
In Closed System S. faecalis: $\Theta g = 37$ min.									

Table 8. GENERATION TIMES OF S. FAECALIS IN MIXED CONTINUOUS CULTURE WITH L. ACIDOPHILUS.

TIME	0.3 k_0m				TIME	k_0m			
	A	A_1	B	B_1		A	A_1	B	B_1
5:30	∞	∞	∞	∞	12:30	∞	∞	∞	∞
7:00	∞	∞	∞	∞	1:30	∞	∞	∞	∞
7:30	∞	∞	∞	∞	2:30	∞	∞	∞	∞
8:00	∞	∞	∞	∞	3:30	∞	∞	∞	∞
In Closed System L. acidophilus: $\Theta g = 107$ min.									

Table 9. GENERATION TIMES OF L. ACIDOPHILUS IN MIXED CONTINUOUS CULTURE WITH S. FAECALIS.

value to study this organism at rates even lower than that of 0.3 $k_o m \times V$. Also the results indicate that flow rates around 0.3 $k_o m \times V$ would be of interest. This could not be done with the infusion - withdrawal pump available at the time of the study. Therefore, it is suggested that in further studies separate pumps should be used in order to have possibilities of a broader variation within the combinations of flow rates employed.

Also constant measurements of pH by means of a pH meter and of oxidation - reduction potentials would be of high value in future investigations. Of course, in the future biochemical analyses must be included in attempt to isolate the agent (agents) causing the interactions, but this will first be of interest when it has been established where the interactions are found and under which conditions. The obvious next step will then be analyses to determine biochemical factors involved.

	Θg	$k_o m = \frac{\ln 2}{\Theta g}$	F >
STREPTOCOCCUS FAECALIS	25.5 MIN.	1.63	135.29 ml/HR
STAPHYLOCOCCUS AUREUS	39.0 MIN.	1.07	88.81 ml/HR
LACTOBACILLUS ACIDOPHILUS	76.4 MIN.	0.55	46.65 ml/HR

Table 10. Values of Θg, $k_o m$ and tolal flow for S. faecalis, S. aureus and L. acidophilus, each in pure culture in closed system with the medium for the combination of these organisms.

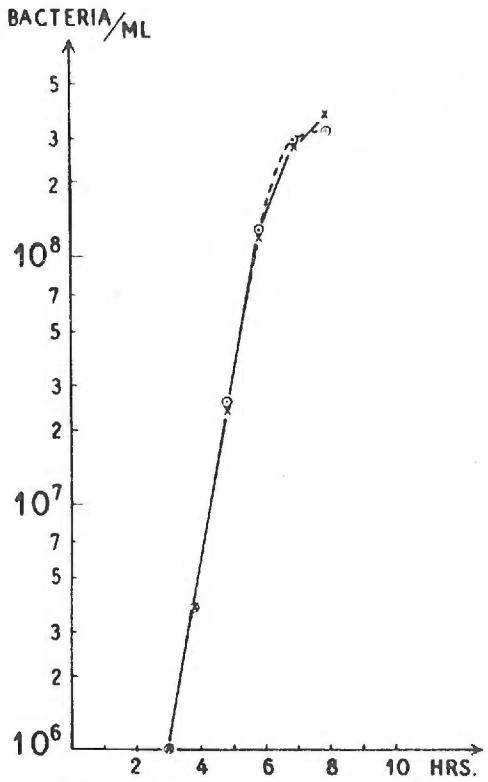


Fig. 20. S. faecalis in closed system. Nutrient: medium for combination of S. faecalis, S. aureus and L acidophilus

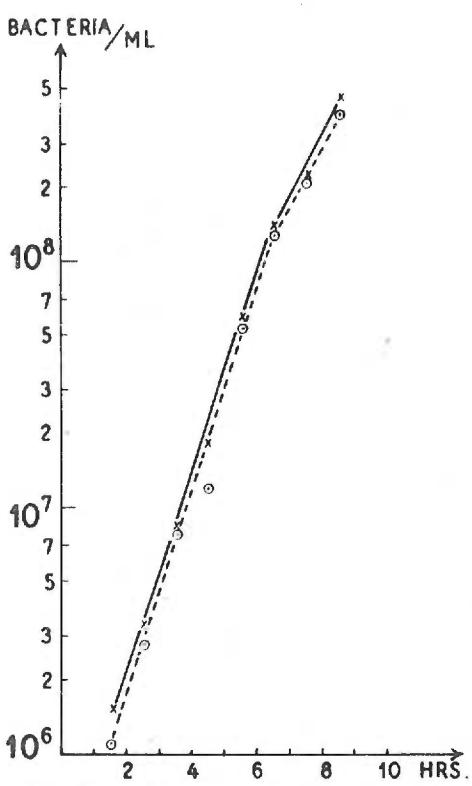


Fig. 21. S. aureus in closed system. Nutrient: medium for combination of S. faecalis, S aureus and L. acidophilus.

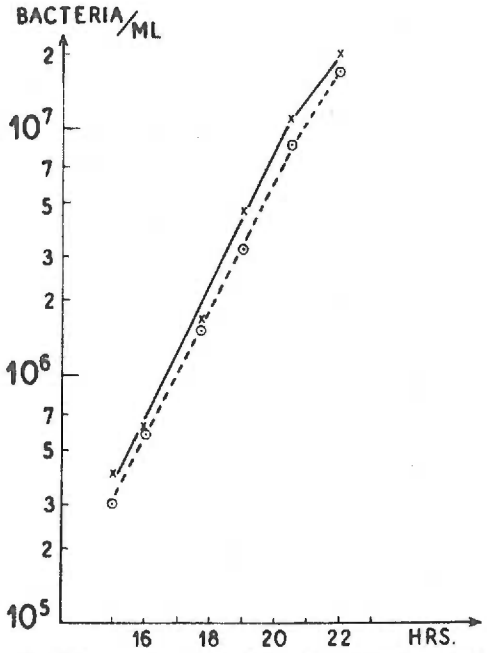


Fig. 22. L. acidophilus in closed system. Nutrient: medium for combination of S. faecalls, S. aureus and L. acidophilus.

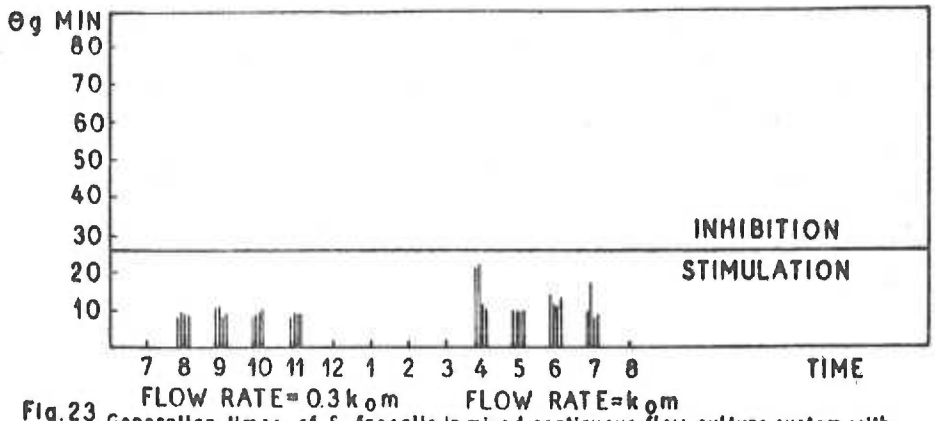


Fig.23 Generation times of S. faecalis in mixed continuous flow culture system with S. aureus and Lactobacillus.

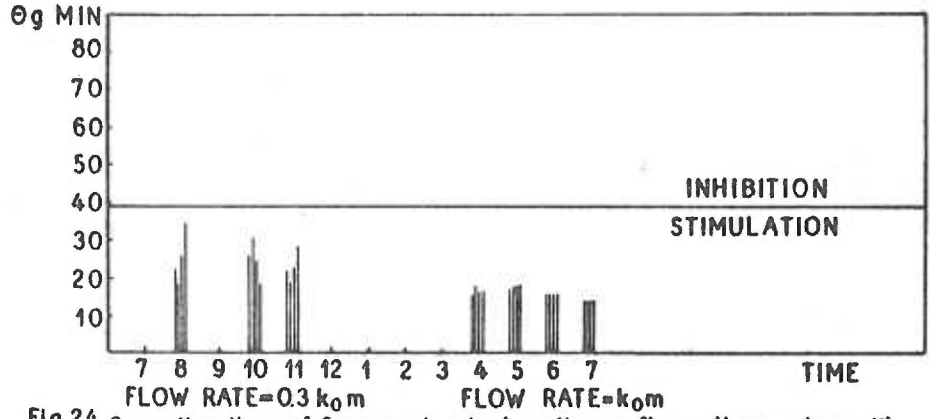


Fig.24 Generation times of S aureus in mixed continuous flow culture system with S. faecalis and L. acidophilus.

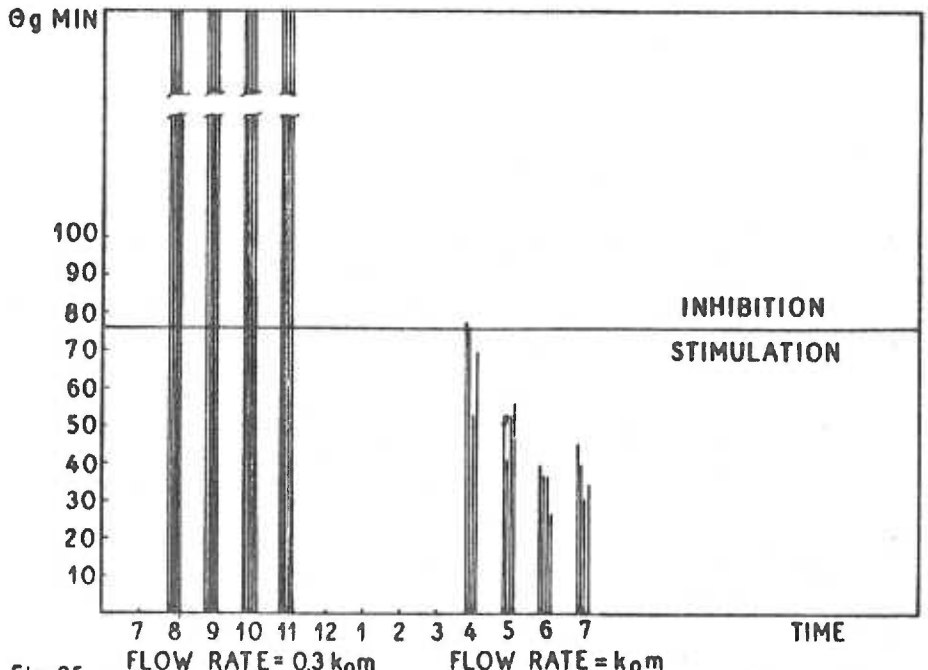


Fig. 25 Generation times of L. acidophilus in mixed continuous flow culture system with S. aureus and S. faecalis.

TIME	0.3 k_0m				TIME	k_0m			
	A	A_1	B	B_1		A	A_1	B	B_1
8:00	8.2	8.5	10.8	8.6	4:00	21.2	22.0	11.1	10.4
9:00	11.3	11.2	8.1	8.5	5:00	9.6	10.1	10.0	9.5
10:00	9.1	8.9	9.2	9.6	6:00	13.5	11.7	10.2	12.5
11:00	8.4	8.6	8.6	9.3	7:00	8.7	17.2	8.4	9.3
In closed system S. faecalis: Θg=25.5 min.									

Table 11. GENERATION TIMES OF S. FAECALIS IN MIXED CONTINUOUS CULTURE WITH S. AUREUS AND L. ACIDOPHILUS.

TIME	0.3 k_0m				TIME	k_0m			
	A	A_1	B	B_1		A	A_1	B	B_1
8:00	15.8	17.7	16.6	16.6	4:00	22.6	18.8	27.4	34.1
9:00	18.1	17.7	18.1	19.0	5:00	∞	∞	∞	∞
10:00	15.8	17.4	15.7	17.4	6:00	27.4	31.1	22.0	18.6
11:00	15.1	15.4	15.3	14.8	7:00	22.0	20.3	22.6	28.7
In closed system S. aureus: Θg = 39 min.									

Table 12. GENERATION TIMES OF S. AUREUS IN MIXED CONTINUOUS CULTURE WITH S. FAECALIS AND L. ACIDOPHILUS.

TIME	0.3 k_0m				TIME	k_0m			
	A	A_1	B	B_1		A	A_1	B	B_1
8:00	∞	∞	∞	∞	4:00	78.5	75.3	54.7	69.5
9:00	∞	∞	∞	∞	5:00	51.6	41.0	53.1	56.4
10:00	∞	∞	∞	∞	6:00	40.1	36.9	36.1	27.3
11:00	86hrs	∞	∞	∞	7:00	43.0	38.4	31.1	34.7
In closed system L. acidophilus: Θg = 76 min.									

Table 13. GENERATION TIMES OF L. ACIDOPHILUS IN MIXED CONTINUOUS CULTURE WITH S. FAECALIS AND S. AUREUS.

DISCUSSION AND SUMMARY

Numerous investigators have suggested interrelationships between the organisms of the indigenous oral flora and between the microflora of the mouth and the biological environment in which it exists (6, 9, 12,20, 42, 43, 44, 45, 49, 55, 56, 60). Many factors are involved in the natural biological balance, which might be disturbed by a minor change in a single factor, resulting in a sequence of changes. The present study has involved an attempt to devise a system usuable for studies of the oral flora under defined conditions approximating those of the mouth. However, at all times it should be remembered that this is an "in vitro" system and that results so obtained cannot necessarily be related to those obtained in an "in vivo" study.

Results from study of pairs. S. faecalis was stimulated by S. aureus independent of flow rates while S. aureus was inhibited at slow flow rate and stimulated weakly at maximum flow rate in the same system.

S. aureus had a very short generation time at 0.3 $k_o m$ as well as at $k_o m$. Generation time was shortest at $k_o m$, when growing with L. acidophilus.

The latter organism was stimulated at $k_o m$ and when flow rate constant equaled 0.3 $k_o m$ the organism appeared to be stimulated after an adaption period. Similarly, S. faecalis was stimulated by L. acidophilus; however, the latter was inhibited both at $k_o m$ and at 0.3 $k_o m$.

Extremely short generation times were obtained in certain instances. In some cases the times indicated were so short that one can wonder if a bacterial cell is able to prepare the necessary protoplasm and divide within so short a time. The possibility of the presence of an artifact of separation of individual cells must be considered. However, the extremely short generation times have been determined often enough to demonstrate reproducibility under the conditions of the experiment.

Results from study of pairs compared with those of three species in combination. When the results from the study of pairs of organisms are compared with those of the combination of all three bacteria the following was concluded. S. faecalis was stimulated by S. aureus and by L. acidophilus both when studied in pairs and when studied with all three organisms in mixture. The shortest generation times were found in paired systems. In mixtures of all three organisms the action of S. aureus and L. acidophilus upon S. faecalis apparently produced a combined stimulating effect with generation times of less than nine minutes in contrast to 25.5 minutes in this medium when grown in pure culture.

S. aureus was stimulated by L. acidophilus but was inhibited significantly by S. faecalis at a slow flow rate. Its generation time appeared irregular when growing with S. faecalis at a rate close to $k_o m \times V$. In the mixed growth cell when S. faecalis and L. acidophilus

were present, S. aureus was stimulated. Thus, the inhibiting action of S. faecalis upon S. aureus at 0.3 $k_o m$ was eliminated and exceeded by the stimulating effect of L. acidophilus at this flow rate.

L. acidophilus was restricted seriously in growth in the presence of S. faecalis. However, in mixture with S. aureus the lactobacillus was stimulated at $k_o m$, whereas at 0.3 $k_o m$ it seemed to need an adaption period before stimulation of growth was evident. Because 0.3 $k_o m$ was used in the first part of the study the above results might indicate a long adaption period was required during rapid exchange of products through the system ($k_o m \times V$). At slow flow rate the lactobacillus was extremely inhibited. Thus, in the case of using fast flow rate near $k_o m \times V$ the stimulating effect produced by the presence of multiplying S. aureus on L. acidophilus must exceed the inhibiting effect by S. faecalis. Where 0.3 $k_o m$ was used both organisms inhibited L. acidophilus.

Genetic aspects. Another very interesting aspect of these studies is the very short generation times (5-6 minutes) that appear to be obtained with S. faecalis when grown in combination with S. aureus. These extremely short generation times may be an artifact caused by mechanical factors, i.e., breaking up of clumps. Notwithstanding this possibility, such results are of exceptional interest and, however explained, the results are the results of a bacterial interaction.

Kjeldgaard (16) has shown that bacteria in continuous flow systems usually have a shorter generation time than when the same organisms are growing in closed systems. These results make us believe that the present device could be useful in finding the shortest generation time possible for different bacteria.

Dental aspects of the study. The results obtained from these investigations can be related to the biological environment to a limited degree for at least two reasons. First of all, this is an artificial system where the microorganisms are taken away from the normal biological system which, alone, makes it dangerous to draw definite conclusions related to the oral milieu. However precisely controlled, test individuals whose total oral environment is known will never be available. One will always have to get as much information as possible from in vitro studies where the investigator tries to perform the experiments under controlled conditions as close to the natural environments as he can manage. This system seems to create a basis for beginning. Secondly, only a few of the bacteria present in the microcosmos of the oral cavity were included in the present study. Interactions from species not yet included might change the equilibrium and final results may bear little relationship to the above.

Results suggest that the system be expanded so that a greater number of oral species can be studied in the mixture. Also, slower flow rates of substrate should be attempted to investigate conditions more representative of the oral environment.

CONCLUSIONS

Within the limitations of the experimental procedures employed, it may be pointed out that the method used has proved to be reliable and sensitive. The most interesting results were obtained at a flow rate of 0.3 $k_o m$. This would indicate that marginal nutritional conditions will yield the most meaningful results and that future studies should be conducted at flow rates less than $k_o m \times V$. Likewise, long-term runs should be performed but during the course of this study they were not possible because of mechanical problems.

The following rationale will justify the use of continuous flow systems in the study of mixtures of oral microorganisms. As indicated by the relative constant numbers of indigenous microorganisms in the saliva, it is proposed that within certain limits $k_o m$ (growth rate constant in the oral environment) must approximate F/V (flow rate of saliva). If this was not true, the species would either be diluted by the saliva flow and the fluid intake by the host or else by multiplying at faster rates a single organism would ultimately exclude all other species in competition for nutrients and for space. In either event the bacterial population of the mouth would vary tremendously. Thus, although it is evident that the numbers of bacteria in the oral cavity do fluctuate the limits of this fluctuation suggest that the net effect is one of a steady state.

S. aureus was included in the study in an attempt to find a reason for the extreme low occurrence of this organism in the saliva, in spite of the fact that it is present in great numbers in the nasal cavity and

on the skin. No effect in this respect was found from S. faecalis and L. acidophilus, whereas in earlier studies by Parker and Snyder (38, 39) it was found that Veillonella alcalescens would inhibit this organism.

The lactobacillus employed in this study was tremendously inhibited when grown with S. faecalis and S. aureus. Conversely, S. faecalis and S. aureus are stimulated. This may be of some importance to the concept of involvement to the etiology of caries.

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APPENDIX 1.

GLYCINE-TELLURITE AGAR

Tryptone	1%
Glycine	1%
Yeast extract	0.5%
Mannitol	0.5%
K_2HPO_4	0.5%
Lithium chloride	0.5%
Agar	2%

Double boil until the agar is melted, cool in water bath until 50° C.

Add 10 ml 2% K_2TeO_3 aseptically.

APPENDIX 2

Substrates used for activation, chemostatic growth and selective counting of *S. faecalis*, *L. acidophilus* and *S. aureus*.

Organism	Activation Medium	Counting Medium	Chemostatic Medium
Streptococcus faecalis	0.1% Trypticase 0.1% Sucrose	Brain-Heart Infusion 6.5% NaCl	0.1% Trypticase 0.01% Sucrose
Lactobacillus acidophilus	1% Trypticase 0.5% Yeast extract 0.1% Sucrose	Rogosa's S Agar pH = 5.5	0.1% Trypticase 0.1% Yeast Extract 0.01% Sucrose
Staphylococcus aureus	0.1% Trypticase 0.01% Thiamine, HCl	Glycine-Tellurite Agar	0.15% KH_2PO_4 0.35% k_2HPO_4 0.01% Mg SO_y $7H_2O$ 0.5% Vitamin Free Casamino Acids 0.1% mg/liter Thiamine HCl

APPENDIX 3

Medium to the mixed growth cell for studied pairs and for combination of three bacterial species.

S. faecalis S. aureus	S. faecalis L. acidophilus	S. aureus L. acidophilus
0.6% trypticase 0.005% Thiamine, HCl 0.005% Sucrose pH = 6.8	1% trypticase 0.25% Yeast extract 0.06% Sucrose pH = 6.8	0.6% trypticase 0.25% Yeast extract 0.05% Sucrose 0.005% Thiamine, HCl pH = 6.8

S. faecalis S. aureus L. acidophilus
1.5% trypticase 0.3% Yeast extract 0.75% Sucrose 0.007% Thiamine, HCl pH = 7.0