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FACTORS AFFECTING THE COLONISATION OF THE GUT BY LACTOBACILLI AND OTHER BACTERIA

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SUMMARY

This article summarises some of the literature concerning the use of probiotics in man and animals and attempts to analyse the difficulties inherent in the current concepts and practices in the field. The mechanisms governing bacterial colonisation of the gastrointestinal tract are reviewed. These are complex and dependent on the macrohabitat, e.g. stomach, small intestine or large intestine, as well as on the microhabitat e.g. lumen, mucus gel or epithelial cell surface. Each of these habitats requires distinct traits on the part of a bacterium to enable it to colonise. For this reason, each of these habitats has a distinct indigenous microflora. Accordingly, a microorganism that is part of a probiotic preparation must also have at least one set of these distinct traits, in order to effectively influence flora in at least one *in vivo* environment. It is therefore unlikely that a single probiotic species, such as lactobacilli, will be able to be effective in more than one *in vivo* environment. Moreover, the normal function of the indigenous microflora requires the presence of a large number (perhaps all) of the indigenous microbial species. Future research must therefore concentrate on the following questions: (1) What is the pathogenesis of the human disorder that is to be affected by the probiotic regimen; at the very least one must know the microenvironment which the offending bacteria inhabit. (2) Which microbial characteristics promote colonisation of this habitat and, consequently, which characteristics must a probiotic bacterium possess for effective competition. It is argued that a broadly effective probiotic must contain a large number of bacterial species. These should be cultivated under physiological conditions resembling a specific intestinal microenvironment, in order to assure that the bacteria would commence multiplication in the gut without a prolonged lag phase (during which they could be washed out without having a chance at colonisation).

INTRODUCTION

A measure that is employed with increasing frequency in human as well as veterinary medicine is the oral administration of bacterial supplements, in the hope of optimising the beneficial and protective functions of the indigenous microflora. The impetus for using bacterial supplements can be traced back to

the ideas of *Metchnikoff* (1907), which were further developed by workers such as *Rettger* et al (1935). The ingestion of preparations such as milk that is either supplemented with, or fermented by lactobacilli or other "beneficial" microorganisms, is widely practised in the Western world, as well as in developing countries. This and related exercises have been described as "probiotics" in the more recent literature. In commenting upon this phenomenon, *Tannock* (1984) writes: "For every article in the scientific literature that claims beneficial results from the ingestion of fermented milk, another article will provide evidence to the contrary. Most of the reported studies have not been adequately controlled, statistical analysis of the results is rarely made, and the conclusions are largely subjective". This subject has been reviewed more recently and in some detail (*Fuller*, 1989; *Freter*, 1992, *Sanders*, 1993a). All of these reviewers emphasise the ambiguities inherent in the relevant scientific literature. Throughout her review Dr. Sanders emphasises that whatever positive evidence exists, it does not actually prove a beneficial effect of specific probiotic regimens, but serves mainly to encourage future, more incisive research. A panel of experts in this and related fields, convened by the California Dairy Research Foundation apparently agreed with this assessment (*Sanders*, 1993b). The evidence available in the published literature clearly shows that it is not possible at the present time to predictably change the function or composition of the indigenous microflora in such a way as to eliminate its harmful effects (e.g. to eradicate those bacterial species likely to cause sepsis after translocation, to invade the urinary tract, give rise to diarrhoea or cause or exacerbate inflammatory bowel disease), and to maximise its beneficial functions (e.g. the protective activity against colonisation by potential pathogens). A better understanding of the mechanisms that control the composition and function of the indigenous microflora is therefore not only of theoretical interest in defining those characteristics that bacteria must possess in order to be able to colonise the gut, but it also has considerable medical importance, as attested to by the program of this seminar.

The uncertainties reviewed above have persisted in spite of almost a century of continuous intensive research in the field. This shortcoming strongly suggests that there are serious flaws in the current concept of probiotics and in much of the research that flowed from those ideas. In this article we will argue that these flaws are a consequence of gross conceptual oversimplifications of the complexities of the gastrointestinal ecosystem and of the diverse effects of the indigenous microflora on other microbes and on the host directly. We plan to demonstrate that it is irrational to expect the administration of a product containing one or a limited number of bacterial species to be predictably effective against a given human disorder in all patients, regardless of the known variability of intestinal flora among individuals. It appears even more irrational to expect such a product to be predictably effective against a variety of human disorders, whether such disorders are clinically similar but with vastly different mechanisms of pathogenesis (e.g. "diarrhoea") or clinically distinct syndromes of equally diverse pathogenesis (e.g. vaginitis, urinary tract infections, sepsis or inflammatory bowel disease). Most research in the field has been of an empirical clinical nature with little emphasis on the detailed pathogenesis of the disorders that the probiotic preparations under study were to alleviate and, consequently, with still less emphasis on the mechanisms by which

the probiotics were supposed to exert their beneficial effects. A variety of such possible mechanisms are summarised in the next paragraph.

In order to make a rational choice of microbial strains to be included in probiotics, and to devise proper regimens for their administration, one needs to consider the following:

1) Is the probiotic intended to reduce the population size of (or to completely eliminate) harmful species? If so, is this to be accomplished by:

a) competition for nutrients or
b) competition for adhesion sites
(i) in the stomach,
(ii) in the small intestine or
(iii) in the large intestine?
(iv) And for *each* of these sites: are the target bacteria multiplying in the lumen, in association with the mucus gel or adherent to epithelial cell surfaces?

Each of these possibilities requires different bacterial characteristics for colonisation and, consequently, must require different characteristics on the part of the probiotic microorganisms for effective competition with colonisation by the target microorganism(s).

2) Is the probiotic intended to modify the intestinal milieu in order to:

a) modify the metabolism of potentially harmful flora in such a way that harmful metabolites (e.g. toxins, carcinogens) are not produced or
b) inactivate or adsorb such harmful metabolites as they are produced?

3) Is the main purpose of the probiotic to stimulate local or (specific or non-specific) systemic immunity after traversing the epithelial barrier (i.e. after translocation), rather than to affect the indigenous flora directly?

Some of these possible mechanisms clearly require viable microbial cells for effective probiotic activity, whereas others may conceivably function with nonviable preparations. Some of these possible mechanisms would require colonisation by the probiotic strains, whereas others may function with strains that merely pass through the gastrointestinal tract without multiplication (and whose presence must be maintained by frequent repeat administration).

This introductory chapter would not be complete, however, without pointing out another irrational view that is frequently embraced in the application of probiotic principles, namely, the choice of a few bacterial species (predominantly lactobacilli and to a lesser extent bifidobacteria, streptococci and others) as effective probiotic agents. Considering the large number of possible mechanisms that must underlay the many diverse probiotic strategies of remedy reviewed above, it is difficult to imagine that a single bacterial species would be effective in all of them. It is much more likely, for example, that interference with the mucosal association of an *E. coli* would be best accomplished by a microorganism that employs identical mechanisms of mucosal association. Only with *in vitro* tests employing unphysiologically high concentrations of competing bacteria could one expect significant non-specific competition with a target strain for adhesion to mammalian cell surfaces. In the same vein, the elimination of an undesirable species of the indigenous flora by means of metabolic competition would be most effective if carried out by a microorganism that employs similar metabolic pathways as the target strain. Why then the widespread preference for lactobacilli as probiotic agents? Part of the answer may lie in the tradition begun by *Metchnikoff* and other early workers, as well as in the simple fact that lactobacilli are easy to culture in large quantities and are rarely the cause of systemic infections in man (even though there are an increasing number

of reports of such incidences as well). More insidious may be the reasoning based on the frequent findings by investigators throughout the past century that a "healthy" or "normal" or "eubiotic" intestinal flora in man and domestic animals is rich in lactobacilli. Many workers seem to have concluded from this that lactobacilli are the agents that bring about such healthful conditions. There is little evidence in the published literature, however, to rule out the alternative view, namely, that a good size population of lactobacilli is merely a consequence of and hence an indicator of a properly functioning intestinal ecosystem (in the same sense that the presence of frogs is sometimes taken as an indicator of a pristine ecosystem, without thereby implying that the frogs are actually responsible for the absence of pollution). If the latter view were correct, then the attempts at establishing a eubiotic flora by replenishing the intestinal lactobacillus populations, would be the equivalent of trying to clean up a polluted refuse dump by releasing frogs or to cure a fever by shaking down the thermometer.

In the remainder of this article we will discuss some of the major mechanisms mentioned above. In view of the interests of the author's laboratory, special emphasis will be given to mechanisms of colonisation, but other important mechanisms will be considered as well. Accordingly, most of the following pages will deal with the flora of the large intestine of the mouse, but the principles to be discussed are likely to apply as well to bacterial populations colonising other body surfaces and other species, including man. As will be discussed, the study of mechanisms underlying intestinal microecology is made exceedingly difficult (1) by the diversity of intestinal microhabitats, each of which imposes peculiar requirements for bacterial colonisation, (2) by the problem of devising *in vitro* model systems that simulate the physiological conditions prevailing in the gut and (3) by the multitude of mechanisms that simultaneously control bacterial population sizes in the gut. These points and some promising experimental approaches will be discussed below under separate subheadings, followed by a brief discussion of the contribution that mathematical modelling can make to overcoming the problems of complexity.

It is important to note that the term colonisation will be used in this article in the common ecological meaning, namely, to denote the presence of a population of microorganisms of constant size, in which the rate of multiplication equals the rate of physical (or other) removal. Consequently, this term will carry no other implications as to possible adherence or other parameters of the *in vivo* habitat.

GENERAL REVIEW OF GASTROINTESTINAL MICROFLORA

As has been discussed by several authors (e.g. *Finegold* et al., 1983), there is now a considerable body of ecological studies available, which describe the bacterial populations in various regions of the human or animal gastrointestinal tract. Nevertheless, much remains to be done along these lines (*Lee*, 1985). The human stomach harbours very few microorganisms, most of which appear to be transients. Spiral shaped organisms, including the potential pathogen *Helicobacter pylori*, may however colonise the gastric mucosa of man (*Hazell* et al., 1986) and animals (*Fox* et al., 1986; *Lee*, 1985),

and a more complex flora may develop in achlorhydria. The proximal small intestine is sparsely populated, with increasing populations found in the ileum. The terminal ileum, in contrast, harbours a large, complex flora that may approximate that of the large intestine. The large intestine harbours the highest concentration of microorganisms found on the human body, with viable counts in the order of 10^{11} colonies per gram of intestinal contents. *Moore* and *Holdeman* (1974) have estimated that there may be over 400 different bacterial species colonising the large intestine. The predominant bacteria are strict anaerobes that require special methods (roll tubes, anaerobic chambers) for cultivation. The population sizes of facultative anaerobes in the large intestine are usually lower by a factor of about 10 to 1,000.

The numerous functions of the intestinal microflora also have been explored extensively. Gnotobiotic techniques have been most valuable for such studies. It appears that there are very few physiological parameters of the human or animal body that are not in some way affected by the presence of the indigenous microflora (reviewed by *Freter*, 1986). Numerous disease states of man or animals are known or suspected to be affected by the metabolic activities, or by an imbalance of the indigenous microflora.

Among the functions of the intestinal microflora that are of greatest importance in human health and disease one must certainly include the protection the flora affords against colonisation by pathogens. The latter phenomenon has been discovered and re-discovered many times since the early days when *Metchnikoff* (1907) thought that the ingestion of lactobacilli would suppress the growth of "putrefactive" bacteria in the gut. Terms such as "bacterial antagonism" (*Freter*, 1956), "bacterial interference" (*Dubos*, 1963), "barrier effect" (*Ducluzeau* et al., 1970) or "colonisation resistance" (*van der Waaij* et al., 1971) have been used to describe and/or quantitate this protective activity. It seems reasonable to assume that this protection against pathogen colonisation is simply a special aspect of, and involves similar mechanisms, as are responsible for the homeostasis of the intestinal flora. In other words, the mechanisms that preserve the balance among intestinal microorganisms and prevent any one or few bacterial species to become dominant, also prevent bacteria that may invade from the environment (including pathogens) from becoming established in the ecosystem of the gut.

Another matter of importance to the subject of this seminar is the fact that the normal intestinal mucosa allows the passage of colloidal particles the size of bacteria across the epithelium into the lamina propria. This is a normal and apparently continuous process. To the knowledge of this author, the phenomenon was first described by *Hirsch* (1906) who showed that the characteristic particles of dietary starch could be found in blood and urine of humans. The appearance of starch granules in these body fluids may begin within minutes after ingestion (*Volkheimer* et al., 1968). In recent times, the subject has been studied most extensively by Berg and co-workers (reviewed in *Deitch* and *Berg*, 1987) and Wells and co-workers (reviewed in *Wells* et al., 1988a, 1988b). It appears to be a passive process on the part of the particle, because even plastic microspheres are readily able to cross the intestinal epithelium (*Wells* et al., 1988a). This phenomenon, often described as "translocation", has two major consequences for human health and disease:

(1) Bacteria entering the lamina propria from the intestinal lumen may proliferate, be carried to other organs and

give rise to sepsis, especially in individuals with impaired systemic immunity. Unfortunately, nothing is known about the most crucial aspect of translocation, namely, the mechanisms by which indigenous bacteria and inert particles traverse the mucosal barrier and whether and how such traversal could be minimised. This is in contrast to the situation with classical pathogens such as salmonellae and shigellae, whose specific mechanisms for penetrating epithelial cells have been studied extensively.

(2) The entry of bacteria from the indigenous microflora into the mucosa and their subsequent translocation to other organs may also stimulate local and/or systemic immunity against these microorganisms as well as against cross-reacting species. For example, feeding milk fermented with some strains of lactobacilli has been shown to increase to a certain extent the resistance of mice to experimental infections with *Salmonella typhimurium*, *Listeria monocytogenes* and other pathogens (cf. *Nader de Macias* et al., 1993), and earlier publications from that group cited therein.

ECOLOGICAL NICHES (MICROHABITATS) OF THE GUT

When bacterial populations colonise mucosal surfaces it is often difficult to determine with certainty whether their habitat is the mucus gel itself, or whether they are more intimately associated with the epithelial cell surfaces. The main reason for this difficulty is the collapse of the highly hydrated mucus gel that occurs during preparation of specimens for electron microscopy. The gel may either coalesce into isolated strands, or it may collapse unto the epithelial cells carrying entrapped bacteria with it, thereby yielding a preparation which may give the impression that the bacteria in the original specimen were also located adjacent to the epithelial surface (*Hill*, 1985; *Rozee* et al., 1982). *Rozee* et al. (1982) developed a method whereby the mucosa is first exposed to anti-mucus antibody before the dehydration step. This method yielded preparations of mouse ileum for electron microscopy in which a continuous mucus blanket was preserved, which contained large entrapped bacterial populations. Another method, i.e. light microscopy of frozen sections, especially when these are stabilised with agents such as methyl cellulose or stained quickly without fixation (*Freter*, et al., 1983), often gives a more realistic estimate of the natural distribution of bacteria on a mucosal surface.

In spite of these uncertainties, it may be useful to distinguish three main micro-habitats along the gastrointestinal tract. The first of these is populated by bacteria that colonise the deep layers of the mucus gel. Spiral shaped bacteria often populate the crypts of the ileum, caecum and colon. *Lee* and co-workers have contributed much to the understanding of these populations (reviewed in *Lee*, 1980; *Lee*, 1985), and have shown that these consist of very different bacteria in various regions of the gut. The common feature of spiral morphology is thought to contribute to the ability to traverse viscous media, such as mucus gel (*Lee*, 1985). In this view the bacteria withstand removal with the mucus flow by active motility directed, perhaps by chemotactic stimuli, towards the bottom of the crypts. In such a situation, special means of attachment to the epithelial surface would not be required for successful colonisation.

The second intestinal habitat for bacterial populations is the surface of

the epithelial cells. Thus, lactobacilli attach to the stomach of mammals and the crop of chicken in a very specific manner, i. e. strains isolated from chicken will not attach to and colonise rats, and vice versa. As mentioned above, some indigenous bacteria of the small and large intestine have developed rather complex and intimate attachment mechanisms to epithelial cells (*Lee*, 1980, 1985). Attachment to the epithelial cells of the small intestine is also the well-known mechanism by which some of the classical enteric pathogens, such as enterotoxigenic *E. coli*, manage to evade removal by the peristaltic activity of the jejunum and ileum, and thus are able to colonise.

Except in acute diarrhoeal diseases, the first two types of micro-habitat discussed above contain relatively sparse populations consisting of one, or only a few different kinds of bacteria. There is, however, a third type which is distinguished by the presence of a dense and complex flora that consists of a large number of different kinds of bacteria. Such habitats include dental plaque, the gingival crevice, the vagina, the crop of birds, the throat, the lower ileum, the caecum and the colon. Bacterial populations in the latter three areas of the intestine form thick layers which are embedded in the mucus gel. It is well known that many of the indigenous species are able to degrade mammalian mucus (reviewed by *Freter*, 1982), and it is therefore uncertain whether the material that surrounds the bacterial populations is indeed entirely of host origin, whether it is partially degraded material of host origin, or whether it represents in part, or entirely, material produced by the bacteria. Be this as it may, it has become apparent in recent years that the mucus gel which overlays the entire epithelium of the gastrointestinal tract forms a third potential habitat for bacteria. Its role in promoting or inhibiting bacterial colonisation has been the subject of much debate (*Freter*, 1982). Some earlier workers considered mucus gel to be a "particle and macromolecule proof coating for cell surfaces" through which bacteria must "bore a channel" by means of special virulence mechanisms. This view has persisted until recent times (*Edwards*, 1978). Studies in the author's laboratory (*Freter* et al., 1981) have implicated the chemotactic attraction of motile bacteria into the mucus gel as a major force aiding such microorganisms in the penetration of the intestinal mucus layer. Inert polystyrene particles in the size range of bacteria or yeast cells also penetrated mucus gel, but at a much slower rate. Superior ability to penetrate mucus gel was correlated with superior ecological fitness: non-chemotactic mutants were rapidly outgrown by their chemotactic parents in rabbit intestinal loops and in gnotobiotic mice. Interestingly, non-motile mutants rapidly outgrew normally motile but non-chemotactic vibrios in gnotobiotic mice. Consequently, motility appeared to confer an ecological burden on the bacteria *in vivo*, unless the motility was also guided by chemotactic stimuli, in which case motility was strikingly advantageous.

More recent unpublished studies from the author's laboratory have extended the work on chemotaxis of vibrios (which populate the small intestine) to bacteria of the predominant, strictly anaerobic flora of the large intestine. When the caeca of mice are removed under strict anaerobiosis in an anaerobic chamber and their contents observed through a microscope located in that chamber, a large majority of the bacteria present show a high rate of motility. A highly motile Gram-positive bacterium (probably of the genus Clostridium) was isolated from the mouse caecum, and a non-chemotactic but normally

motile (smooth swimming) mutant selected. This mutant showed significantly reduced ability to enter the mucosa of the mouse caecum, and was not able to colonise the mouse large intestine when the chemotactic parent was also present. Interestingly, no evidence of positive chemotaxis could be demonstrated with the parent strain, nor with a number of other strictly anaerobic bacteria isolated from the mouse caecum. On the other hand, all motile anaerobes tested showed strong negative chemotaxis against short chain fatty acids (e.g. acetic, butyric, propionic acids). It is therefore entirely possible that many anaerobes that populate the large intestine are indeed incapable of positive chemotaxis, and that they are driven towards the mucosa, their natural habitat, by a gradient of negative taxins such as the short chain fatty acids which accumulate in the lumen as the metabolic endproducts of many indigenous microbial species.

EXPERIMENTAL MODELS FOR THE STUDY OF INTESTINAL MICROECOLOGY

It is well known that most bacteria, even those species not indigenous to the intestinal flora, are able to colonise germfree animals, whereas colonisation of conventional animals or healthy people is usually difficult to achieve experimentally (*Freter*, 1983). One must conclude therefore, that some of the major mechanisms that control the composition of the indigenous microflora of the large intestine are based on the interactions among the numerous microbial species present. Since it is difficult to study such interactions in the intact animal where homeostatic mechanisms necessary for survival of the host limit the range of feasible experimental manipulations, most early (and even contemporary) investigators resorted to working with *in vitro* models of bacterial interactions. This approach creates a serious problem because *in vitro* models cannot a priori be relied upon to reflect the mechanisms by which microorganisms interact *in vivo*. This is a consequence of the well - known ecological principle that the nature of interactions among different populations, whether these be microorganisms or higher forms, depends to a large extent on the nature of the habitat in which these interactions take place. Many instances have been recorded in the literature documenting the lack of correlation between microbial interactions among different kinds of bacteria observed *in vitro*, and the interactions among these same bacteria in the gut of intact animals (discussed in more detail by *Freter*, 1983, 1992).

A reasonably well-established exception to the inadequacy of most *in vitro* models appears to be the anaerobic continuous flow (CF) culture, which can duplicate the numerical relationships among the complex flora of the large intestine, as well as reproduce bacterial interactions as they occur in the large intestine (*Hentges* and *Freter*, 1962; *Freter* et al., 1973, 1983; *Veilleux* and *Rowland*, 1981; *Edwards* et al., 1985; *Wilson* and *Freter*, 1986; *Bernhardt* et al., 1987, 1988). The mere fact that CF cultures are able to maintain a natural balance among the numerous species populating the large intestine, is a strong argument supporting the conclusion that the ecological control mechanisms in CF cultures are similar to those operating *in vivo*, because it is difficult to imagine two different sets of mechanisms which fortuitously would bring

about similar equilibria in populations as complex as those of the indigenous microflora of the large intestine. This somewhat surprising distinction of anaerobic CF cultures appears to be due to a number of features which this culture device shares with the mammalian large intestine. The most obvious of these is the physical feature of continuous flow. In addition, the CF culture shares with the large intestine the dense and complex populations associated with the wall. These adherent populations are critical for the ability of a CF culture to simulate the intestinal ecosystem (*Freter* et al., 1983). Even though the mechanisms of adhesion to the glass walls of a CF culture device must differ from those on the gut wall, most bacteria in the thick layers of mucosa-associated bacteria obviously adhere to each other rather than to the mucosal surface. Consequently, adhesion to the wall in a CF culture may indeed resemble that of the intestine, except for the innermost layer of bacteria which adhere to the glass.

It is important to realise that the study of bacterial interactions *in vitro* or in gnotobiotic animals serves at least one useful function, regardless of the model system employed, namely, to identify mechanisms of bacterial interaction which potentially might be involved in the control of bacterial populations in the gut. In this manner a large number of mechanisms have been identified by which one bacterium may inhibit the growth of another under physiological conditions which, when realised in the intestine, would still be compatible with life of the animal. These include changes in oxidation-reduction potential, acidity, the presence of inhibitory substances such as bacteriocins, fatty acids, hydrogen sulphide and deconjugated bile salts, as well as competition for nutrients, competition for adhesion sites, and local immunity (reviewed by *Savage*, 1977). In attempting to evaluate this range of possibilities and the likelihood that any one of these is important in controlling the microbial populations in the gut, it will be useful to first contemplate in the next several paragraphs some of the details of intestinal microecology which any hypotheses concerning control mechanism must be able to explain (and which will be explained by the mathematical model described later in this article):

Most important here is the fact that the more than 400 species which comprise the flora of the lower ileum and large intestine show a relatively constant distribution of species, i.e. all of these coexist without one or a few displacing the others. Considering this high diversity, it is impossible to conceive of a single mechanism that would be sufficient to bring about such a complex equilibrium.

A second important property of the intestinal microflora that must be considered here, is its stability. Stability implies, of course, that microorganisms (including pathogens) that enter the gut from the environment are prevented from colonising it. Even if the invader strain is of a type indigenous to the gut (e.g. a recently isolated *E. coli*), its ingestion will rarely result in colonisation of the host (reviewed by *Freter*, 1983). Nevertheless, the same *E. coli* strain will usually colonise well and become a part of the indigenous flora when it is introduced first, i.e. as a monocontaminant into germfree mice, and when the indigenous microflora is then implanted afterwards. In this type of experiment the implanted indigenous flora functions normally, i. e. it does inhibit the colonisation of bacteria that are ingested later. Nevertheless, the *E. coli* strain that was introduced first maintains a constant population at a density typical of indigenous *E. coli* in conventional mice (*Freter* et al., 1983).

In this connection one may ask an important question, namely, which bacteria are actually involved in the control of the flora, i.e. which are the important species that confer colonisation resistance by means of their antagonistic activities? In an earlier study from this laboratory (*Freter* and *Abrams*, 1972) it was shown that it required 95 anaerobic bacteria to reduce an *E. coli* population to levels comparable to those found in conventionalised "normal" mice (i. e. animals associated with caecal homogenate from conventional mice). The important lesson to be derived here is that the control of bacterial populations (indigenous as well as pathogens) is unlikely to be the function of a single indigenous species, such as *Lactobacillus*, but rather requires the activity of many (and possibly all) indigenous bacteria.

The third and last important feature of intestinal microecology that must be considered here, concerns the dynamics of bacterial growth in the large intestine. The optimal growth rate of *E. coli* in a monoassociated mouse is similar to that in a broth culture, with a doubling time of approximately 20 minutes (*Freter* et al., 1983). In contrast, the mean retention time of contents in the large intestine of the mouse is much longer, in the order of 3 hours (ibid.), and bacteria with a doubling time of less than 3 hours would soon form populations of infinite size in the gut. Consequently, most and possibly all bacteria inhabiting the gut appear to multiply at a rate that is considerably slower than the maximum rate which they could sustain in a monoassociated animal or *in vitro*. One must conclude, therefore, that the physiological conditions of the large intestine are much less than optimal for bacterial multiplication, a shortcoming which may be due to the lack of nutrients, the presence of inhibitors, or both. Most importantly, it appears that the control of growth rates and population sizes of bacteria in the large intestine are mostly due to activities of the indigenous microflora itself, rather than being attributable to host defences.

Earlier studies from this author's laboratory took advantage of the special properties of anaerobic CF cultures, discussed above, and have identified a number of mechanisms that control the growth and colonisation of bacteria in CF cultures of mouse caecal flora (*Freter* et al., 1983). These include (a) the presence of H_2S, which appears to restrict the utilisation of certain nutritional substrates by the bacteria; (b) competition for nutrients of the kind which can be used as carbon and energy sources under the prevailing conditions of strict anaerobiosis and in the presence of H_2S; (c) association of the indigenous microflora with the mucus layer of the intestinal wall; (d) the prolonged lag phase that invading bacteria entering the environment of the CF culture, or of the large intestine, must undergo. This prolongation of the lag phase appears to be caused, at least in part, by the presence of short-chain fatty acids, which are metabolic endproducts of the predominant anaerobic flora. Under the prevailing physiological conditions in the large intestine, lag phases of newly introduced bacteria may extend over several days and, for this reason, newly introduced bacteria may be washed out of a CF culture before they can begin multiplication. In contrast, invading bacteria that were introduced into a CF culture of mouse intestinal flora after having been harvested from a donor CF culture of mouse intestinal flora, were able to multiply without a lag phase and were able to successfully colonise the recipient CF culture (*Freter*, unpublished). The possible relevance for this seminar would be the expectation that bacteria in probiotic preparations may indeed be able

to colonise a patient when they are harvested from cultures that physiologically resemble the specific microenvironment of the gut that is to be targeted.

ANALYSING COMPLEXITIES AND DEVELOPING HYPOTHESES BY MEANS OF A MATHEMATICAL MODEL

Although the above analysis of control mechanisms in CF cultures has identified several that are likely to operate also in the intestine, there is no indication of the relative importance of these mechanisms to the overall balance. For example, it is easy to determine *in vitro* that a given bacterium is able to adhere to epithelial cells. This determination gives no information, however, as to the governing parameters of this adhesion (e.g. the rate constants of adhesion and elution), and consequently does nor permit one to decide whether this adhesion is indeed of a nature that would allow the bacterium to colonise a certain area of the gut or whether it is merely an *in vitro* phenomenon. It is likely, furthermore, that the relative importance of various mechanisms changes in different circumstances. For example, very slow growth rates of bacteria are likely to be of less decisive impact on populations that adhere to the wall, as compared to populations residing in the lumen (*Freter* et al., 1983). The role of adhesion in the large intestine also needs further explanation. As described above, CF cultures failed to simulate bacterial interactions in the mouse large intestine when adhesion of the flora to the wall of the culture vessel was prevented. This is surprising because of the long mean retention time of contents in CF cultures and in the large intestine. It is well known that the peristaltic movements in the small intestine are so rapid that bacteria can only colonise by adhering to the gut wall. That is not the case in the large intestine, however, and bacterial populations could well compensate for the slow washout rate by their potentially much faster rates of multiplication. Why then the need for adherent bacterial populations in the large intestine?

In order to evaluate the above - mentioned questions, a mathematical model was developed which describes the fate of an "invader" strain that is being swallowed (or inoculated into an established CF culture of the indigenous microflora) where it competes with an already established "resident" strain that may or may not belong to the same species. In this approach, the rest of the indigenous flora is not described in detail, but is regarded as a part of the intestinal environment (which, as discussed above, is indeed defined to a large extent by the metabolic and other activities of the indigenous flora). The mathematical model has the following properties (*Freter*, 1983):

(1) Resident and invader strains have exactly the same physiological characteristics. (2) Both residents and invaders compete for the same adhesion sites on the wall of the gut or CF culture. (3) Both residents and invaders compete for the same limiting nutrient. (4) Offspring of adherent bacteria occupy additional adhesion sites until most sites are filled. Thereafter, daughter cells of adherent bacteria are shed into the lumen. (5) Adhesion of bacteria is reversible, governed by rate constants for adhesion and elution in a mass action type of relation.

Application of this model to the ecology of mouse large intestinal flora in CF cultures led to a hypothesis (*Freter*, 1983), which states that the populations of most indigenous bacteria of the large

intestine are controlled by substrate competition, i.e. that each indigenous species is more efficient than the rest in utilising one (or a few) of the many nutritional substances that are present in the gut. Such substrates may be components of the diet as well as mucopolysaccharides and cell debris of host origin, all of which may be partially modified by some components of the flora. The hypothesis further holds that the function of the system is modified by the presence of inhibitors (such as H_2S). A colonising species or strain is successful when it can realise the highest rate of multiplication at the lowest concentration of a particular nutrient, as compared to all of its actual and potential competitors. This would explain the presence of several hundred bacterial species in the large intestine, each colonising at constant population sizes.

The hypothesis finally postulates that the regulation described above is modified further by the effects of bacterial association with the wall. Residents (which are already associated with the wall) are washed out of the system at a rate which is much slower than that for freely suspended material. Consequently, the population of adherent residents will expand until the concentration of the limiting nutrient is reduced to the point where it will support a rate of multiplication of the residents which just balances their rate of elution. For this reason residents can form constant populations at nutrient concentrations which are too low to support a growth rate that would be adequate to maintain a constant population of the invaders, which, at least initially, are all suspended and therefore are washed out more rapidly. Consequently, invaders are at a relative disadvantage to residents even if the two populations have identical physiological properties. This explains the colonisation resistance exerted by the indigenous microflora. It also resolves the apparent paradox mentioned above namely, the necessity for adhesion in the large intestine where the rate of bacterial elimination is much slower than the maximal growth rate of the bacteria. The mathematical model shows that association of the indigenous microflora with the mucosa is necessary for stability, by creating conditions (i.e. low nutrient concentrations) that are adverse to colonisation by invaders. An invader, therefore, can colonise only if it is able to rapidly find sites for association with the mucosa. This may be possible if the invader can adhere to sites different from those occupied by the resident, or if it is more efficient in associating at the same sites, i.e. if its rate constant of adhesion is higher, or if its rate constant of elution is lower than that of the resident. These interactions among nutrient concentrations, growth rates, and rates of adhesion and elution also explain the observed phenomenon that a bacterium which cannot colonise a CF culture harbouring an established indigenous flora, can nevertheless become a resident strain if it is implanted before the indigenous flora. In the latter case, the bacterium can colonise the adhesion sites for which it has an affinity and subsequently can reduce the concentration of the limiting nutrient(s) to a level that only allows mucosa-associated bacteria to colonise (*Freter*, 1983). Looking at this same phenomenon from the perspective of an invader bacterium, it is not only important that an invader can adhere to (or associate with) the intestinal wall, it is also important that this adhesion proceed at a rapid rate, such that association with the mucosa can be accomplished and multiplication can begin before the invader is washed out by peristalsis.

CONCLUSIONS

The above described hypotheses concerning the mechanisms controlling the mouse large intestinal flora could not have been formulated without a close co-ordination between experimental studies and an evaluation of the experimental data by the mathematical model. The hypotheses still incorporate a number of assumptions, and certainly will have to be expanded and modified in details as more evidence becomes available. Nevertheless they do provide a unified picture of this complex ecosystem which heretofore had not been available, and which explains much of what is known of the behaviour of the system.

What can these hypotheses contribute to an understanding and possible solution of the practical problems discussed in the introduction to this article, namely the possibility that physicians should be able to predictably alter the indigenous microflora of patients such as to minimise the detrimental effects of this flora and to maximise its beneficial functions? If, according to the hypotheses, each type of bacterium in the ecosystem does indeed depend for colonisation on its ability to associate with the mucosa, and on its ability to be most efficient in utilising one or a few limiting nutrients, then the development of an effective resistance to colonisation by unwanted bacteria requires that the resident populations collectively are able to react with all available adhesion sites, and that they are able to utilise all available nutrients. No single bacterial strain or species can be expected to monopolise the efficient utilisation of all nutrients and to occupy all potential adhesion sites. Consequently, the homeostasis of a "healthy" flora and the establishment of resistance to colonisation by bacteria from the environment is likely to require the presence of a complex flora. It is unlikely, therefore, that the deliberate administration of single bacterial cultures to man or animals will accomplish the desired end of significantly decreasing the likelihood of colonisation by undesirable microorganisms. One must conclude, therefore, that the administration of a complex flora would be much more promising. Indeed, several workers have already employed this approach in man and animals (reviewed in *Freter*, 1992). As discussed above, it is very difficult for newly introduced microorganisms to replace an already established flora. For this reason, non-absorbable broad spectrum antibiotics would have to be given to suppress at least most of the indigenous flora prior to the administration (either by mouth or as an enema) of the complex replacement flora. The replacement flora could be maintained in CF cultures, which would eliminate the danger of inadvertently transmitting human viruses or protozoan parasites (which do not propagate in CF cultures). Bacterial strains to be included in the complex replacement flora could be selected for such characteristics as high growth rates at minimal nutrient concentrations in a physiological environment resembling the intestine, as well as for maximal ability to associate with the mucosa at a rapid rate by means of chemotaxis, adhesins, etc. The bacterial strains in a probiotic preparation should be produced by culture methods that physiologically resemble the conditions of the gut microenvironment that is to be targeted (e.g. the mucus layer in the ileum). Molecular genetic techniques may be used to minimise the ability of component strains of the complex replacement flora to cause sepsis after translocation from the gut.

It needs to be emphasised that a realisation of the above mentioned ideas re-

quires a considerable amount of basic research. We need to know considerably more about how indigenous flora interacts with the various microenvironments of the human body. For obvious ethical reasons, such studies can only be undertaken in animal models. While it is true that there are differences among mammalian species and man, animal model experiments are nevertheless indispensable in delineating the kinds of host-bacterium interactions that occur in nature. Once these have been studied in some detail, counterparts of such interactions can be searched for in human patients. In view of the complexities of host - bacterium interactions, some of which were outlined in this article, and considering our present ignorance of the basic nature of many of them, one must seriously question the usefulness of the currently practised approach of seeking "effective" strains of certain species and empirically studying their influence on human disorders of varied and often uncertain pathogenesis.

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LITERATURE

Bernhardt, H., Knoke, M., Bootz T., and Zschiesche M.: Simulierung des intestinalen mikrobiellen Overgrowth durch continuierliche Kultur. Dtsch. Z. Verdau.-Stoffwechs. Krankh 47, 261-267 (1987).

Bernhardt, H., Knoke, M., and Bootz, T.: Simulation of the intestinal microflora in a continuous flow culture. In Bengt Gustavsson Symp.: The Regulatory and protective role of the normal microflora. Stockton Press, Stockholm (1988)

Deitch, E.A., and Berg, R.: Bacterial translocation from the gut: A mechanism of infection. J. Burn Care Rehabil. 8, 475-482 (1987).

Dubos, R.: Staphylococci and infection immunity. Am. J. Dis. Child. 105, 643-645 (1963).

Ducluzeau, R., Bellier, M., and Raibaud, P.: Transit digestif de divers inoculums bacteriens introduits 'Per os' chez des souris axeniques or 'holoxeniques' (conventionelles): Effects antagoniste de la microflore du tractus gastro-intestinal. Zbl. Bacteriol. I. Abt. Orig. 213, 533-548 (1970).

Edwards, P.A.W.: Is mucus a selective barrier to macromolecules? Br. Med. Bull. 34, 55-56 (1978).

Edwards, C.A., Duerden, B.I., and Read, N.W.: Metabolism of mixed human colonic bacteria in a continuous culture mimicking the human cecal contents. Gastroenterol. 88, 1903-1909 (1985).

Feinegold, S.M., Sutter, V.L., and Mathisen, G.E.: Normal indigenous intestinal flora. In: Human intestinal flora in health and disease (Ed.: Hentges, D.J.). Academic Press, Boca Raton, 3-31 (1983).

Fox, J.G., Edvise, B.M., Cabot, N., Beaucage, C., and Murphy, J.C.: Isolation of Campylobacter-like organisms from gastric mucosa in the ferret. Am. J. Vet. Res. 47, 236-239 (1986).

Freter, R.: Experimental enteric *Shigella* and *Vibrio* infections in mice and guinea pigs. J. Exper. Med. 104, 411-418 (1956).

Freter, R.: Bacterial association with the mucus gel system of the gut. In: Microbiology-1982 (Ed.: Schlessinger, D.). American Society for Microbiology, Washington, D.C., 278-281 (1982).

Freter, R.: Mechanisms that control the microflora in the large intestine. In: Human intestinal flora in health and disease (Ed.: Hentges, D.J.). Academic Press, Boca Raton, 33-54 (1983).

Freter, R.: Gnotobiotic and germfree animal systems, In: Bacteria in nature, a treatise on the interaction of bacteria and their habitats (Eds.: Leadbetter, E.R., and Poindexter, J.S.). Plenum Publ. Corp., New York., 205-227 (1986).

Freter, R.: Factors affecting the microecology of the gut. In: Probiotics (Ed.: Fuller, R.). Chapman and Hall, London, 111-143 (1992).

Freter, R., and Abrams, G.D.: Function of various intestinal bacteria in converting germfree mice to the normal state. Infect. Immun. 6, 119-126 (1972).

Freter, R., Abrams, G.D., and Aranki, A.: Patterns of interaction in gnotobiotic mice of a synthetic 'normal' intestinal flora. In: Germfree Research. Biological effects of gnotobiotic environments (Ed.: Heneghan, J.B.). Academic Press, London, 429-433 (1973).

Freter, R., Brickner, H., Botney, M., Cleven, D., and Aranki, A.: Mechanisms which control bacterial populations in continuous flow culture models of mouse large intestinal flora. Infect. Immun. 39, 676-685 (1983).

Freter, R., Brickner, H., Fekete, J., and Vickerman, M.M.: Survival and implantation of *E. coli* in the intestinal tract. Infect. Immun. 39, 686-703 (1983).

Freter, R., and O'Brien, P.C.M.: The role of chemotaxis in the association of motile bacteria with intestinal mucosa: Chemotactic responses of *Vibrio cholerae* and description of motile non-chemotactic mutants. Infect. Immun. 34, 215-221 (1981).

Freter, R., and O'Brien, P.C.M.: The role of chemotaxis in the association of motile bacteria with intestinal mucosa: Fitness and virulence of nonchemotactic *Vibrio cholerae* mutants in infant mice. Infect. Immun. 34, 222-233 (1981).

Freter, R., and O'Brien, P.C.M.: The role of chemotaxis in the association of motile bacteria with intestinal mucosa: *In vitro* studies. Infect. Immun. 34, 234-240 (1981).

Freter, R., Stauffer, E., Cleven, D., Holdeman, L.V., and Moore, W.E.C.: Continuous flow cultures as *in vitro* models of the ecology of large intestinal flora. Infect. Immun. 39, 666-675 (1983).

Fuller, R.: Probiotics in man and animals. J. Appl. Bacteriol. 66, 365-378 (1989).

Hazell, S.A., Lee, A., Brady, L., and Hennessy, W.: *Campylobacter pyloridis* and gastritis: Association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J. Infect. Dis. 153, 658-663 (1986).

Hentges, D.J., and Freter, R.: *In vivo* and *in vitro* antagonism of intestinal bacteria against *Shigella flexneri*. I. Correlation between various tests. J. Infect. Dis. 110, 30-37 (1962).

Hill, R.H.: Prevention of adhesion by indigenous bacteria to rabbit cecum epithelium by a barrier of microvesicles. Infect. Immun. 47, 540-543 (1985).

Hirsch, R.: Ueber das Vorkommen von Staerkekoernern im Blut und im Urin. Z. Exp. Path. Ther. 3, 390-395 (1906).

Lee, A.: Normal flora of animal intestinal surfaces. In: Adsorption of microorganisms to surfaces. (Eds.: Bitton, G., and Marshall, K.C.). John Wiley & Sons, New York., 145-173 (1980).

Lee, A.: Neglected niches, the microbial ecology of the gastrointestinal tract. Adv. Microb. Ecology 8, 115-162 (1985).

Metchnikoff, E.: The prolongation of life. Optimistic studies. Heinemann, London, 151-183 (1907).

Moore, W.E.C., and Holdeman, V.L.: Human fecal flora: The normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. 27, 961-979 (1974).

Nader de Macias, M.E., Romero, N.C., Apella, M.C., Gonzales, S.N., and Oliver, G.: Prevention of infections produced by *Escherichia coli* and *Listeria monocytogenes* by feeding milk fermented with lactobacilli. J. Food Protection 56, 401-405 (1993).

Rettger, L.F., Levy, M.N., Weinstein, L., and Weiss, J.E.: *Lactobacillus acidophilus* & its therapeutic applications. Yale University Press, New Haven (1935).

Rozee, K.R., Cooper, D., Lam, K., and Costerton, J.W.: Microbial flora of the mouse ileum mucous layer and epithelial surface. Appl. Envir. Microbiol. 43, 1451-1463 (1982).

Sanders, M.E.: Effect of consumption of lactic cultures on human health. Adv. Food and Nutrition Res. 37, 67-130 (1993a).

Sanders, M.E.: Summary of conclusions from consensus panel of experts onnhgealth at-

tributes of lactic cultures: significance to fluid milk products containing cultures. J. Dairy Sci. 76, 1819-1828 (1993b).

Savage, D.C.: Microbial ecology of the gastrointestinal tract. Ann. Rev. Microbiol. 31, 107-133 (1977).

Tannock, G.W.: Control of gastrointestinal pathogens by normal flora. In: Current perspectives in microbial ecology (Eds.: Klug, M.J., and Reddy, C.A.). American Society for Microbiology, Washington, D.C., 374-382 (1984).

van der Waaij, D., Berghuis-de Vries, J.M., and Lekkerkerk, J.E.C.: Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J. Hyg. 69, 405-511 (1971).

Veilleux, B.G., and Rowland, I.: Simulation of the rat intestinal ecosystem using a two-stage continuous culture system. J. Gen. Microbiol. 123: 103-115 (1981).

Volkheimer, G., Schulz, F.H., Aurich, I., Strauch, S., Beuthin, K., and Wendlandt, H.: Persorption of particles. Digestion 1, 78-80 (1968).

Wells, C.L., Maddaus, M.A., Erlandsen, S.L., and Simmons, R.L.: Evidence for the phagocytic transport of intestinal particles in dogs and rats. Infect. Immun. 56, 278-282 (1988a).

Wells, C.L., Maddaus, M.A., and Simmons, R.L.: Proposed mechanisms for the translocation of intestinal bacteria. Rev. Infect. Dis. 10, 958-979 (1988b).

Wells, C.L., Jechorek, R.P., and Erlandsen, S.L.: Evidence for the translocation of *Enterococcus faecalis* across the mouse intestinal tract. J. Infect. Dis. 162, 82-90 (1990).

Wilson, K.H., and Freter, R.: Interactions of *Clostridium difficile* and *E. coli* with microfloras in continuous flow cultures and gnotobiotic mice. Infect. Immun. 54, 354-358 (1986).