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8. PROBIOTICS: PROSPECTS OF USE IN OPPORTUNISTIC INFECTIONS

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PROBIOTICS: THEIR DEVELOPMENT AND USE

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SUMMARY

There is some confusion about the meaning of the word "probiotic" which can mean different things to different people. A new, more general definition is proposed which covers the use of live microbial supplements for animals, plants and foods. The basis of the probiotic concept is briefly reviewed. While the protective effect of the gut microflora is beyond question, the specific microorganisms responsible for the effect is less well defined. The results of field trials with probiotics often seem to be variable. The reasons which might account for this apparent lack of consistency are discussed.

INTRODUCTION

The habit of consuming fermented milks has a long history going back hundreds of years. PreChristian cave drawings show transfer of fermented milk to fresh milk with the intention of maintaining the ferment. These early producers were probably aware of the preservative effect that fermentation had on the milk which would otherwise have been wasted. In this way, it could be said to have had an indirect health benefit. What they were not aware of was the benefit derived from the ingestion of the microorganisms responsible for the fermentation.

It was not until the end of the last century that the consumption of fermented milk was related to health. This relationship was given a scientific basis by *Metchnikoff*, who attempted to reproduce the effect obtained with fermented milks by using specific cultures of bacteria isolated from those milks. The interest which his researches attracted led to a fashionable habit of consuming fermented milks which has persisted to this day and in recent years, has increased with the advent of the bioyoghurts which now grace the shelves of all our supermarkets.

In this paper, It will be discussed what we mean by probiotics, the way in which they have evolved and been developed and the reasons for the variations which bedevil the assessment of the effectiveness of probiotics as beneficial food supplements.

DEFINITION

The word "probiotic" has had several different meanings over the years and even at present it is used by different people to mean different things. It is, therefore, important at this stage, before we embark on any further discussions,

to consider what it is we mean by probiotics.

The word was first used by *Lilley* and *Stillwell* in 1965 to describe substances secreted by one microorganism which stimulated the growth of another. It described, as the derivation of the word would demand, an agent which was the exact opposite of antibiotic. However, the word used in this sense was never widely adopted and in 1971 *Sperti* used it to describe tissue extracts which stimulated microbial growth.

The first use of the word to describe a microbial feed/food supplement was by *Parker* in 1974. He defined it as "organisms and substances which contribute to intestinal microbial balance". Later (*Fuller*, 1989) this was modified to read "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". This modified definition removed the word "substances" which could have included antibiotics. It also stressed the viable nature of the preparations. There is now evidence that, in order to be effective, microorganisms should be viable.

It is five years since this definition was proposed and it may be that there is now a case for revising it. One criticism is that it is not wide enough and should embrace not only effects on the intestinal flora but other groups of the indigenous microflora, such as occur in the vagina and respiratory tract. *Havenaar* and *Huis in 't Veld* (1992) have attempted to extend the definition in such a way. They propose that probiotics should be defined as "a mono or mixed culture of live microorganisms which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora".

Two years later, even this definition may not be adequate. For example, should we include microbial stimulants such as the bifidogenic factors, and should we also extend it still further to include effects obtained in plants and even in food? With this in mind, the following definition was suggested for consideration and discussion during the seminar that formed the basis for this monograph: "A probiotic is a preparation consisting of live microorganisms or microbial stimulants which affects the indigenous microflora of the recipient animal, plant or food in a beneficial way".

All these definitions refer to microorganisms and would include bacteria, yeasts, fungi, viruses and bacteriophages. All these groups of organisms with the exception of the animal and plant viruses have been shown to have beneficial effects when administered to animals. The microorganisms included as probiotics are usually assumed to be non-pathogenic components of the normal microflora, such as the lactic acid bacteria. However, there is good evidence that non-pathogenic variants of pathogenic species can operate in much the same way as traditional probiotics. For example, avirulent mutants of *Escherichia coli*, *Clostridium difficile*, and *Salmonella typhimurium* can also protect against infection by the respective virulent parent strain. Are these what we understand as probiotics?

Perhaps the animal preparations should be separated from those applicable to plants and food. It might help to dispense with the word "probiotic" altogether, since it is becoming increasingly difficult to define. Some scientists already prefer to talk about "microbial feed supplements". Time will tell whether the term "probiotics" is too firmly ensconced to be dispensed with or whether a more easily defined term can be adopted to replace it.

At present, probiotics include the traditional fermented milks such as yoghurt which do not contain organisms which originate from the animal indige-

nous microflora. The justification is historic because fermented milk was what first stimulated interest in the possible beneficial effects of microbial supplements and led to the development of preparations which contained microorganisms derived from the animal gut with the potential to improve the nutrition and health of the consumer.

DEVELOPMENT

The scientific study of probiotics began with *Metchnikoff*. Towards the end of the last century there grew up a feeling that fermented milks were good for you. *Metchnikoff* generated the theory that under normal circumstances the microflora of the lower gut was having an adverse effect - the so-called autointoxication. He proposed that the ingestion of saccharolytic organisms in the form of fermented milk was reversing the effects of the proteolytic organisms which caused the autointoxication. His approach was encapsulated in his book "Essais optimistes" published in 1907. In it he says: "A reader who has little knowledge of such matters may be surprised by my recommendation to absorb large quantities of microbes, as a general belief is that microbes are harmful. This belief is erroneous. There are many useful microbes, amongst which the lactic bacilli have an honourable place".

Although this was said nearly ninety years ago, it would still surprise many non-microbiologists to learn that microorganisms are not necessarily bad for you.

Metchnikoff isolated microorganisms from fermented milk and used them in feeding trials. One isolate he called the Bulgarian bacillus. Although it is impossible to be certain, this was probably the same as the organism which later was classified as *Lactobacillus bulgaricus* and is now called *L. delbrueckii* subsp. *bulgaricus* which, together with *Streptococcus salivarius* subsp. *thermophilus*, is used to ferment milk to yoghurt.

After *Metchnikoff*'s death in 1916, the centre of activity moved from Europe to North America. Workers in the USA questioned the use of *L. delbrueckii* subsp. *bulgaricus*. They reasoned that since the effect was being manifested in the gut, it would be better to use an organism which originated from that site. At that time *L. acidophilus* was the lactic acid bacterium which was most commonly isolated from the gut. When this was used in human feeding trials it gave encouraging results in treatment of constipation. However, not all the trials with this organism gave positive results.

By the 1930's, treatment of infectious diseases was dominated by chemotherapy and by 1940 penicillin had been developed. Although this tended to suppress the interest in probiotics, it was responsible in an indirect way for increasing our understanding of the benefit that might be derived from the gut microflora. In the late 1940's, it was shown that low levels of antibiotics in the feed of animals improved their growth, implying that there was in the gut a population of microorganisms which were adversely affecting their development. It was important to know how this was operating and it stimulated research into the composition of the gut microflora. It soon became obvious that there were many species of lactic acid bacteria other than *L. acidophilus* present in the gut. As a result a variety of different species of the genera *Lactobacillus, Streptococcus* and *Bifidobacterium* were incorporated into probiotic

preparations. These are still the three most commonly used probiotic organisms.

The current status of the probiotic effect and its scientific basis are reviewed in a recently published book (*Fuller*, 1992).

THE CONCEPT

The protective effect of the gut microflora is beyond question. It has been studied by many groups throughout the world and has been described as bacterial antagonism (*Freter*, 1956), bacterial interference (*Dubos*, 1963), barrier effect (*Ducluzeau* et al., 1970), colonisation resistance (*van der Waaij* et al., 1971) and competitive exclusion (*Lloyd* et al., 1977).

There is strong experimental evidence to support the belief that certain components of the gut microflora are involved in protection of the host against infectious disease.

If animals are reared in the complete absence of a microflora they become more susceptible to disease; the protection can be restored by colonising them with a gut microflora derived from the same animal species. The protective effect has also been studied by administering antibiotics by mouth to compromise the protective effect and get some indication of the types of microorganism responsible. The human studies have been confusing because the results have incriminated the anaerobes (*van der Waaij* et al., 1972), anaerobes and aerobes (*Wells* et al., 1988) and others have produced evidence to show that the anaerobes are not involved (*Gorbach* et al., 1988). Experimental studies in chickens (*Mead* and *Impey*, 1987) and rats (*Wilson* and *Freter*, 1986) suggest that a large number of strains are necessary for the full protective effect. A recent paper, however, was able to reproduce protection against *Campylobacter jejuni* by dosing chickens with only three species of bacteria - *Citrobacter diversus, Klebsiella pneumoniae* and *E.coli* (*Schoeni* and *Wong*, 1994).

However, we should regard this result with some caution until it has been tested against other strains of *C. jejuni.* Previous work (*Barrow* and *Tucker*, 1986) showed that protection could be obtained with three strains of *E. coli* but further work revealed that this was a specific effect which was only active against the strain of *Salmonella. typhimurium* used in the original trials.

One well documented condition in humans is the post antibiotic diarrhoea caused by *Clostridium difficile.* This condition is amenable to treatment with an enema produced from faeces of a healthy human adult (*Eiseman* et al., 1958).

There is, therefore, no doubt that the gut microflora is at least partly responsible for the resistance to intestinal infections. Various factors other than antibiotic therapy can affect the composition of the microflora. For example, high standards of hygiene, which are absolutely necessary and cannot be relaxed, may inhibit transfer of protective microorganisms from mother to offspring. In extreme cases, like children delivered by caesarian section into incubators, there is evidence that the flora can be deficient in lactobacilli (*Hall* et al., 1990). In such cases, early administration of a multi-strain probiotic might improve their resistance to disease.

FACTORS AFFECTING PROBIOTIC RESPONSE

The many claims that have been made for probiotics include suppression of diarrhoea, anti-tumour activity, stimulation of immunity, relief of lactose intolerance and growth stimulation of farm animals. Evidence in support of many of these claims will be presented in other papers in this monograph. It is not, therefore, intended to dwell on the details of the results obtained. Unfortunately, the results often appear inconsistent and variable and provide a stick with which to beat the probiotic concept. These differences are often more apparent than real. There are, in many cases, reasons for these apparent inconsistencies and these will now be considered in some detail. The result obtained in a probiotic trial could be affected by the following factors.

The type of organism in the probiotic

While it is perhaps obvious that the results obtained with two different species of microorganism cannot be compared, what is not always appreciated is that it is possible, for two different strains of the same species, to yield different results. Thus, although two probiotics may contain *L. acidophilus* the two strains used may differ in some apparently minor way which may still be significant in producing the probiotic effect. For example, the ability to adhere to the gut epithelium may not be related to any changes which affect the classification of the strain. It is well known that epithelial adhesion is a host specific effect and an *L. acidophilus* strain isolated from the chicken gut will not adhere to the epithelium of the pig. Other colonisation factors such as acid resistance and bile tolerance may vary within species; assuming these features are important in determining the probiotic response, their variability will be reflected in the effect obtained.

Method of production

Even if the two strains being used for production of the probiotic are identical, the way in which they are prepared can cause variation in the results. The effect of production methods on viability of the probiotic has been discussed at some length by *Lauland* (1994). Obvious differences in presentations such as whether the probiotic is a powder or a liquid suspension are readily appreciated. (*Ushe* and *Nagy*, 1985).

However, what is not so obvious are the changes which may be induced by the way in which the probiotic organism is grown and harvested. For example, the carbohydrate source in the growth medium can affect the ability to adhere to the gut epithelium of chickens and the adhesion capacity also changes during the growth cycle (*Fuller*, 1975).

Method of administration

The probiotic may be administered to the host animal in a variety of ways. It may be given as a powder, tablets, liquid suspension, capsule, paste or spray. Moreover, the amount and interval between doses may vary. Probiotics may be given only once or periodically at daily or weekly intervals. Little is known about the minimum dose required for the probiotic effect but trials in rats, humans and pigs indicate that the effect falls off after administration of the probiotic ceases (*Cole* and *Fuller*, 1984; *Goldin* and *Gorbach*, 1984). It therefore seems very likely that the effect obtained will be affected by the amount and frequency of dosing.

Viability of the preparation

Probiotic preparations cannot always be relied upon to contain the number of viable organisms stated on the label. In

many of the published studies, the viability of the preparation used was not checked and negative results may be due to insufficient viable cells being present in the probiotic. In a survey of commercially available probiotic preparations, *Gilliland* (1981) found that the viable count varied greatly and three of the fifteen preparations tested had no viable lactobacilli. Sometimes lactobacilli other than the one listed on the label were present.

Condition of host

Pollman et al (l980) obtained a better probiotic response in starter than he did with growing-finishing pigs. It is widely thought that the earlier the probiotic supplement is introduced the more effective it will be. Certainly, during the early stages of life the gut microflora is in an unstable condition and organisms given by mouth are likely to find a niche which they can occupy. In mammals, the suckling period is different in many ways from the post-weaning period and administration of microbial supplements will survive or not depending on whether they find the environment suitable. Differences have also been observed in the response to fungal probiotics in lactating and non-lactating cows. While lactobacillus probiotics may be more effective in calves, they are of limited use in adult ruminants where fungal probiotics are more effective.

Condition of gut microflora

A probiotic will only be effective if the animal receiving it is subject to the adverse affect which the probiotic reverses. The most obvious example of this is an infectious disease but it is less apparent when probiotics are used to stimulate the growth of farm animals. If, like antibiotics, probiotics stimulate growth by antagonising a growth depressing organism present in the gut, then it will follow that if the organism is not present, no growth stimulation will occur. It has been shown that antibiotics are not effective in stimulating the growth of chickens if they are housed in clean surrounding.

It is not surprising that with all this scope for differences between experiments, that some inconsistency exits. Unfortunately, the information necessary to make judgements about the reasons for variability are often not given in the reports. When they are, they are frequently ignored and comparisons made between two sets of results that are not comparable. It may be that the conditions under which a probiotic will have its maximum effect are very strictly defined and that only if these conditions are met will it appear positive. There is no doubt that because of this the results with probiotics appear to be inconsistent but close scrutiny of the results leads one to conclude that with the right probiotic, administered in the right way at the right time we can expect to obtain a beneficial effect. More knowledge of how probiotics work and the optimal methods for administration will enable us to select more active strains and administer them in a fashion that will make the results more consistent and predictable.

It is expected that this monograph will contribute to the understanding of what these conditions are likely to be and will help to suggest ways in which future research endeavours may be aimed at establishing the basic facts about the probiotic effect that will lead to the development of new and more effective probiotic preparations.

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THE MICROECOLOGY OF LACTIC ACID-PRODUCING BACTERIA IN THE GASTROINTESTINAL TRACT

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SUMMARY

Laboratory animals colonised with a conventional microflora that lacks a specific group of lactic acid-producing bacteria provide tools to determine the influence of the latter microbes on host animal characteristics. Nucleic acid probes and ribotyping (a method that reveals DNA restriction fragment length polymorphism) permit the detection, enumeration, or differentiation of strains of lactic acid bacteria cultured from digestive tract samples. Animal experimentation and the use of molecular epidemiological methods facilitate the study of the interactions of lactic acid bacteria with their environment (their microecology).

INTRODUCTION

Lactic acid-producing bacteria (lactic acid bacteria) are represented among the members of the normal microflora and inhabit the digestive tract of many animal species including human beings, pigs, fowl and rodents (*Tannock*, 1990). Although many of the species comprising the microflora, including some Gram-negative facultative and obligate anaerobes, produce lactic acid during the fermentation of carbohydrates, it is usual to restrict reference of the lactic acid bacteria to Gram-positive species. Microflora members belonging to the genera *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, and *Enterococcus* all produce lactic acid as a major fermentation product and are Gram-positive bacteria and will therefore be considered to be lactic acid bacteria for the purposes of this review (*Sneath*, 1986).

Numerous species of lactic acid bacteria can be detected in the digestive tract (Table 1), but their prevalence and distribution varies according to the animal species with which they are associated. Lactobacilli, for example, are present in large numbers throughout the gastrointestinal tract of pigs, fowl and rodents, probably due to the ability of certain lactobacillus strains to adhere to, and colonise, epithelial surfaces in proximal regions of the tract in these animals (*Tannock*, 1990; *Tannock*, 1992a). A layer of lactobacillus cells forms on the epithelial surface, and bacteria shed from the layer continually inoculate the digesta which moves through the remainder of the digestive tract (*Tannock* et al., 1990). In contrast, the normal microflora of the human gastrointestinal tract is confined to the distal small bowel and the large bowel, and bifidobacteria are more numerous than lactobacilli (*Finegold* et al., 1983).

The acquisition of the streptococcal, enterococcal, lactobacillus and bifidobacterial microflora by neonates has been studied in some detail. In general, lactic acid bacteria are among the pio-

neer organisms of the digestive tract and are numerous in the tract from soon after birth (hatching). The acquisition of the microflora is complex, however, as exemplified by the lactobacillus succession demonstrated to occur in the gastrointestinal tract of piglets (*Tannock* et al., 1990). Streptococci remain as one of the numerically dominant populations of the oral cavity, at least in humans and rodents, but enterococcus populations decrease in size dramatically in the bowel once obligately anaerobic bacteria become established (*Schaedler* et al., 1965; *Stark* and *Lee*, 1982; *Marsh* and *Martin*, 1984). Bifidobacteria are numerous in the gastrointestinal tract of humans throughout life, as are lactobacilli in the case of rodents, fowl and pigs (*Savage*, 1977; *Stark* and *Lee*, 1982; *Finegold* et al., 1983; *Tannock* et al., 1990). Even exposure to large numbers of lactic acid bacteria only early in life might have lifelong consequences for the host: a phenomenon described as "biological freudianism" by *Dubos* and colleagues (1966).

Lactic acid bacteria figure prominently in discussions of probiotics, and are commonly included in commercially available preparations (*Fuller*, 1989). The interest in lactic acid bacteria as probiotic agents has historical (*Metchnikoff*, 1907; *Rettger*, 1935), industrial (dairy industry; *Renner*, 1991), and scientific bases (*Fuller*, 1989). In the latter case, tantalising data and concepts as to how these bacteria can benefit the health of humans and other animals have been reported (*Fuller*, 1989; *Sanders*, 1993). There are two concepts as to how lactic acid bacteria may be used as probiotics:

(a) the lactic acid bacteria are administered, directly or indirectly, by mouth (drench, mixed with food, sprayed on eggs/chickens). The bacteria used as inoculum colonise the digestive tract so that, even after cessation of the administration of the probiotic, the bacteria continue to form part of the normal microflora. The probiotic strains interact with the host directly, or interact with other members of the microflora which, in turn, influence the host. The interactions alter the intestinal milieu biochemically, physiologically, or immunologically, conferring benefits on the host.
(b) as above, but the probiotic strains do not colonise and must be administered continuously in food.

The study of probiotics is therefore the study of the relationships between the lactic acid bacteria and their environment. In other words, the study of their ecology. Since it is a study concerning microbes, the term "microecology" is appropriate. In my opinion, there are two important questions to resolve in relation to the microecology of the lactic acid bacteria.

(a) Do they influence the biochemistry of the intestinal milieu? Without convincing evidence that they do, further studies on the development of the probiotic concept would be less attractive.
(b) How can the fate of specific strains in the complex normal microflora of the digestive tract be monitored? If this cannot be done reliably, interactions between the lactic acid bacteria and other microbes cannot be followed, nor can the fate of probiotic strains administered to the animal be ascertained.

Research in my laboratory has concentrated on answering these two questions. The influence of lactic acid bacteria on the biochemistry of the intestinal tract can best be studied using laboratory animals in whose digestive tract these bacteria normally attain a high

Table 1: Lactic acid bacteria detected in the digestive tract of humans, pigs, and rodents

Bifidobacterium adolescentis	*Lactobacillus acidophilus*	*Streptococcus acidominimus*
B. animalis	*L. amylovorus*	*S. agalactiae*
B. bifidum	*L. brevis*	*S. anginosus*
B. boum	*L. buchneri*	*S. avium*
B. breve	*L. casei*	*S. bovis*
B. choerinum	*L. catenaforme*	*S. constellatus*
B. dentium	*L. crispatus*	*S. cricetus*
B. eriksonii	*L. delbrueckii*	*S. crista*
B. globosum	*L. fermentum*	*S. durans*
B. infantis	*L. gasseri*	*S. equinus*
B. longum	*L. jensenii*	*S. equisimilis*
B. pseudolongum	*L. johnsonii*	*S. gordonii*
B. suis	*L. helveticus*	*S. intermedius*
B. thermophilum	*L. lactis*	*S. intestinalis*
	L. murinus	*S. mitis*
	L. plantarum	*S. morbillorum*
	L. reuteri	*S. mutans*
Enterococcus avium	*L. rogosae*	*S. oralis*
E. faecalis	*L. ruminis*	*S. rattus*
E. faecium	*L. salivarius*	*S. salivarius*
		S. sanguis
		S. sobrinus
		S. uberis

ee: *Finegold* et al., 1983; *Slots* and *Taubman*, 1992; and *Tannock*, 1992a.

population level. The derivation of animals that lack one of the lactic acid groups, but which continued to harbour an otherwise complex microflora, has permitted the comparison of biochemical characteristics of the intestine with those of animals harbouring the specific lactic acid bacteria. Groups of animals that differed only in the presence or absence of certain lactic acid bacteria in the normal microflora have thus been compared. Any differences observed between the animals in these comparisons must have been due to influences exerted by the lactic acid bacteria since all other factors were constant. Mice have been chosen as the laboratory animal system for this work since lactobacilli, enterococci, and streptococci are represented among the normal microflora of their digestive tract. The mice can be maintained in isolators using gnotobiotic methods so that experiments can be carried out under microbiologically-constant conditions. The following murine colonies have been derived using BALB/c mice.

Lactobacillus-, enterococcus-, streptococcus-free mice (LF mice)

These animals also lack some undetermined members of the large bowel microflora since caecal size is larger than normal in these mice (*Tannock* and *Archibald*, 1984). The LF animals are being used to study the colonisation of the oral cavity by *Streptococcus gordonii* strains (*Loach* et al, 1994).

Reconstituted lactobacillus-free mice (RLF mice)

In comparison to conventional mice, these animals harbour a functionally complete normal microflora of the gastrointestinal tract except that lactobacilli are absent (*Tannock* et al., 1988). Work with these animals has demonstrated that lactobacilli influence markedly the biochemistry of the intestinal contents in terms of enzymes produced either by the lactobacilli or other members of the microflora.

(a) Bile salt hydrolase activity detected in RLF mice was reduced by 86% in the distal small bowel compared to RLF animals whose gastrointestinal tract was intentionally colonised by lactobacilli (RLFL mice). The activity was 98% lower in the absence of lactobacilli and enterococci (74% in the caecum). Bile salt hydrolase activities were lower in the ileum and caecum of LF mice intentionally colonised by enterococci compared to LF animals colonised by lactobacilli. Bile salt hydrolase activity in the duodenum, jejunum, ileum, and caecum of RLFL mice was similar to that in samples from the intestinal tract of conventional mice. It was concluded from these studies that lactobacilli are the main contributors to total bile salt hydrolase activity in the murine intestinal tract (*Tannock* et al., 1989).

(b) Conjugated and unconjugated bile acid concentrations were measured in small bowel contents and portal sera collected from mice with (RLFL) or without (RLF) lactobacilli as gastrointestinal inhabitants. The major portion of the bile acids in the small bowel contents of RLFL mice was unconjugated (67.9%) in contrast to RLF animals where a smaller portion of the bile acids was unconjugated (23.5%). This study demonstrated that bile salt hydrolase produced by lactobacilli is active under the conditions prevailing in the proximal small bowel of mice (*Tannock* et al., 1994).

(c) Azoreductase activity in the caecum of RLF mice was compared to that of RLFL mice. Azoreductase activity was 31% lower in the caecum of the mice colonised by lactobacilli (RLFL) (*McConnell* and *Tannock*, 1991a).

(d) A comparison was made of ß-glucuronidase activity in the caecal contents of RLF and RLFL mice. Male RLF mice had about 52% more caecal ß-glucuronidase activity than did their female counterparts. Colonisation of male mice by lactobacilli reduced the ß-glucuronidase activity to that of female mice (*McConnell* and *Tannock*, 1993a).

Lactobacilli have not, however, been observed to influence biochemical characteristics of murine origin.

(a) Alkaline phosphatase and phosphodiesterase I activities of duodenal enterocytes harvested from RLF and RLFL mice were determined. The presence of lactobacilli as members of the digestive tract microflora did not influence the two

enzyme activities (*McConnell* and *Tannock*, 1993b).

(b) The total concentration of cholesterol was measured in sera collected from RLF and RLFL mice. Strains of lactobacilli that "assimilated" cholesterol *in vitro* were included in the lactobacillus microflora of the RLFL animals. Female mice had lower cholesterol concentrations than male animals. The presence of lactobacilli in the gastrointestinal tract did not influence the total concentration of cholesterol or the amount associated with the heavy density lipoprotein fraction in the serum (*Tannock* and *McConnell*, 1994).

Enterococcus-free mice (EF mice)

These animals harbour a functionally complete gastrointestinal microflora except that enterococci are absent. The mice have been used to study the transfer of genetic determinants (R plasmids) between lactobacilli and enterococci (*McConnell* et al., 1991b).

NUCLEIC ACID PROBES AND RIBOTYPING FOR THE DETECTION, ENUMERATION OR DIFFERENTIATION OF SPECIFIC STRAINS OF LACTOBACILLI IN DIGESTIVE TRACT SAMPLES

The application of molecular biological techniques to microecological studies has provided sensitive tools by which interactions between bacterial genera, species and even strains can be monitored. While the use of nucleic acid probes and the polymerase chain reaction can, in theory, be used to detect directly the presence of unique nucleotide base sequences in samples from Nature, selective culture of the appropriate group of bacteria from digestive tract samples is currently more satisfactory (*Tenover*, 1988; *Greisen* et al., 1994). The selectively cultured bacteria can then be probed to detect and enumerate the specific strain of interest. Probes derived from plasmid DNA have proved useful in microbiologically controlled experimental settings, but probes that have chromosomal targets might be preferable considering that plasmids may be transferred between strains or lost by the bacterial host. It should be noted, however, that cryptic (absence of associated phenotype) plasmids of lactobacilli are maintained stably both *in vitro* and *in vivo* (*Tannock* et al., 1990; *Rodtong* and *Tannock*, 1993). Plasmids encoding antibiotic resistance are less stably maintained (*Tannock* et al., 1994).

Detection of epithelium-associated lactobacilli using plasmid-based probes

Biotin-labelled DNA probes prepared from whole plasmids (5.5 and 4.8 kb pr) of two lactobacillus strains (*Lactobacillus delbrueckii* strain 21 and *Lactobacillus reuteri* strain 100-23) were used to detect homologous bacteria in microtome-cryostat-prepared sections of murine forestomach. The forestomach sections were incubated on nylon filter membranes placed on agar plates and, after lysis of the lactobacillus cells and denaturation of their DNA, were used in hybridisation experiments with the strain-specific DNA probes. Hybridisation of the probes to membranes containing sections from lactobacillus-free mice did not occur. The probes detected the presence of homologous strains of lactobacilli in sections cut from the forestomachs of mice harbouring one or both of the strains (*Tannock*, 1989).

Detection of a lactobacillus strain in porcine gastric contents using a plasmid-based probe

A plasmid (about 50 kb pr) was used as a DNA probe to enumerate, by colony hybridisation, a strain of *Lactobacillus fermentum* in the stomach contents of eight piglets. The population sizes obtained by colony hybridisation were in agreement with estimated levels calculated on the basis of plasmid profiling of colonies isolated at random from the total lactobacillus population (*Tannock* et al, 1992).

Detection and enumeration of *Lactobacillus acidophilus* strain O in piglet digestive tract samples.

Four DNA probes were derived that hybridised specifically to DNA from *Lactobacillus acidophilus* strain O. The probes were constructed by randomly cloning lactobacillus DNA in plasmid vector pBR322. Two of the probes (pSR1 and pSR2) were composed of vector and plasmid DNA inserts (3.6 and 1.6 kb pr respectively); the others (pSR3 and pSR4) were composed of vector and chromosomally derived inserts (6.9 and 1.4 kb pr respectively). The probes were used to enumerate, by colony hybridisation, strain O in digestive tract samples collected from piglets inoculated 24 hours previously with a culture of the strain. The probes did not hybridise to DNA from lactobacilli inhabiting the digestive tract of uninoculated piglets. Strain O made up about 10% of the total lactobacillus population of the pars oesophagea and about 20% of the population in other digestive tract samples (*Rodtong* et al, 1993).

Differentiation of lactobacillus strains using ribotyping

Ribotyping is a method by which restriction fragment length polymorphism of DNA can be detected in bacterial strains of the same or different species of a particular genus. The method uses ribosomal RNA sequences (rRNA) as the basis for a broad-spectrum probe for strain differentiation. DNA extracted from bacterial isolates is digested with appropriate restriction endonucleases, the resulting fragments of DNA are separated in an agarose electrophoretic gel, transferred to a hybridisation membrane, and probed with a radiolabelled nucleotide sequence derived from that of *Escherichia coli* rRNA or rRNA from the bacterial genus being tested. Bacteria have multiple copies of rRNA operons in their chromosome, thus several DNA restriction fragments containing rRNA gene sequences are observed after hybridisation with the labelled probe. Ribosomal RNA sequences from *E. coli* can be used as a probe for any bacterial species because there are highly conserved rRNA sequences throughout the bacterial world. Fragment length polymorphism revealed by comparison of the hybridisation patterns permits differentiation between bacterial strains (*Stull* et al., 1988). In our study, fifty four lactobacillus strains were differentiated by ribotyping. The stability of ribotypes characteristic of four strains of lactobacilli inhabiting the digestive tract of mice were investigated. One of four isolates of *Lactobacillus delbrueckii* strain 21, which had been associated with mice for 22 months, had an altered ribotype. It is recommended, therefore, that more than one restriction endonuclease be used to characterise each strain. *Eco*RI and *Hind*III are appropriate since they generate DNA fragments of a suitable range of molecular weights and have markedly different restriction recognition sequences. Thus any base substitution in one restriction site is unlikely to alter the ribotype generated by the other enzyme. There would thus be less likelihood of mistaking a slightly

altered ribotype generated by one enzyme as evidence of a new strain (*Rodtong* and *Tannock*, 1993).

POTENTIAL MICROECOLOGICAL STUDIES THAT COULD UTILISE THE LABORATORY ANIMAL SYSTEMS AND MOLECULAR BIOLOGICAL TOOLS

(a) Determine the molecular mechanisms by which lactic acid bacteria influence the production of potentially toxic substances in the intestinal tract (azoreductase, ß-glucuronidase). This work would be significant because the products generated by the enzymic activities may contribute to the aetiology of cancer of the large bowel. Azoreductases, for example, catalyse the reductive cleavage of azo bonds in dyes used in the food industry as colouring agents. A wide variety of bacterial species inhabiting the large bowel synthesise azoreductases, and it has been postulated that these enzymes can mediate the formation of mutagenic, aromatic amines in the intestine (*Rowland*, 1981). ß-glucuronidase catalyses the cleavage of glucuronic acid molecules from glucuronides entering the intestinal tract in bile and might lead to the reactivation of potentially carcinogenic molecules that had been detoxified by the formation of glucuronides in the liver (*Drasar* and *Barrow*, 1985). The major producers of ß-glucuronidase in the large bowel are *E. coli* and obligately anaerobic bacteria such as *Bacteroides* and *Clostridium* species (*Drasar* and *Hill*, 1974).

(b) Observe the interactions between bacteriocin-producing and appropriately susceptible and resistant strains of lactic acid bacteria in the digestive tract. The importance of bacteriocins in digestive tract colonisation is still an unresolved issue.

(c) Determine the molecular nature of the mechanisms by which lactobacilli adhere to epithelial surfaces. Current opinion is that both proteins and carbohydrate moieties are involved, but the precise mechanisms have not been described and the universality of the theoretical models that have been proposed is unknown (*Brooker* and *Fuller*, 1975; *Barrow* et al, 1980; *Conway* and *Kjelleberg*, 1989).

(d) Monitor the composition of the human normal microflora using ribotyping (strain-specific) and nucleic acid probes (species-specific) in relation to dietary modification. The normal microflora of the large bowel of humans appears to be stable at the level of bacterial genera and species composition. In other words, for any one human subject, the same bacterial genera and species can be detected in the same numbers from faecal samples collected over weeks or months (*Finegold* et al., 1983). The stability of the microflora at the level of bacterial strains, however, has not been evaluated. The strains representing a given bacterial species in the microflora may change temporally. Indeed, there is some evidence based on the serotyping of *E. coli* isolates that such an instability exists (*Mason* and *Richardson*, 1981). The stability of lactic acid bacterial populations, or that of obligately anaerobic species, has not been investigated. Knowledge of

the stability of the microflora is necessary if future studies of dietary supplementation or dietary modification are to be judged worthwhile. A totally stable microflora, for example, is likely to be refractory to modification by the ingestion of lactic acid bacteria in probiotics. Changes in enzyme activities in large bowel contents that have been observed during the course of studies involving dietary modification could be due to induction of enzyme synthesis by permanent microbial inhabitants or to the loss or acquisition of bacterial strains of appropriate biochemical characteristics. These possibilities can only be investigated if technology adequate for detailed analysis of the normal microflora is available.

(e) Test the ability of genetically modified lactic acid bacteria to deliver novel products to the intestinal milieu. An example of such an approach would be to genetically modify a lactic acid bacterium so that its cells synthesised an immunogen characteristic of a specific pathogen. Colonisation of the digestive tract with the recombinant lactic acid bacterial strain could result in continuous exposure of the intestinal mucosa to the immunogen so that secretory IgA antibodies would be synthesised by the host animal. This immunological stimulation by the modified lactic acid bacterium could result in immunity to the pathogen, since the antibodies would prevent binding of pathogenic cells or toxins to epithelial surfaces lining the digestive tract (*Tannock*, 1992b).

(f) Observe the influence of lactic acid bacteria on immunological phenomena. Comparison of the immunological properties of germfree and conventional animals has shown that the normal microflora is a major source of antigenic material that non-specifically stimulates the immunological tissues of the host. This effect of the microflora, particularly the activation of macrophages, is thought to enhance aspects of resistance that are important during the early stages of infection (*Gordon* and *Pesti*, 1971). Numerous reports of non-specific stimulation of the immunological system by lactic acid bacteria have appeared in the literature, but the biological relevance of the results requires investigation (*Tannock*, 1991).

CONCLUSION

Laboratory animal systems and the application of molecular biological techniques permit microecological studies in which phenomena relating to the digestive tract ecosystem can be investigated. The information derived from these studies is of significance in the design, development, use and in enhancing the credibility of probiotics.

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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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MODIFICATION OF GUT FLORA METABOLISM BY PROBIOTICS AND OLIGOSACCHARIDES

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SUMMARY

Gut bacterial metabolic activities play an important role in influencing the toxic effects of many ingested chemicals by activating them to more toxic, genotoxic or carcinogenic derivatives. Modification of the activity of these enzymes by ingestion of lactic acid bacteria or of oligosaccharides which stimulate the growth of lactic acid bacteria in the colon could have beneficial effects for the host.

INTRODUCTION

The metabolic activities of the gut microflora, particularly towards ingested xenobiotics, can have wide-ranging implications for the health of the host, resulting in both detrimental and beneficial effects (Table 1; *Rowland* et al., 1985; *Rowland* and *Walker*, 1983).

Metabolic reactions occurring in the gut can have consequences both locally, on the gut mucosa, or systemically. For example, amines and phenols generated by gut bacteria from amino acids, can have effects on the central nervous system, the vascular system and potentially on tumorigenesis in various organs of the body (*Bakke*, 1969; *Boultwell* and *Bosch*, 1969; *Drasar* and *Hill*, 1974).

The region of the gut which harbours the greatest number of bacteria is the colon, indeed other areas of the human gastrointestinal tract are very sparsely populated (*Drasar*, 1988). This does not mean, however , that only poorly absorbed ingested chemicals encounter the colonic flora. Substances, and their metabolites, may partition across the intestinal wall from the blood or may reach the colon after excretion in the bile (see below). Thus there is ample opportunity for a wide variety of materials in diet to encounter, and be metabolised by, the colonic microflora.

Table 1: Some health implications of gut flora metabolism

1. Production of toxic, carcinogenic or mutagenic metabolites from substances derived from diet or produced endogenously
2. Detoxification of dietary toxicants
3. Enterohepatic circulation of drugs, food additives and steroids

Table 2: Some bacterial enzymes that generate toxic, genotoxic, or carcinogenic products

Enzyme	Substrates
ß-Glycosidase	Plant glycosides • Rutin • Franguloside
Nitroreductase	Nitro compounds • Dinitrotoluene
Azoreductase	Azo compounds • Benzidine-based dyes
ß-Glucuronidase	Biliary glucuronides • Benzo(a)pyrene • IQ • Benzidine
IQ "hydratase-dehydrogenase"	IQ, MeIQ
Nitrate/nitrite reductases	Nitrate, nitrite
Bile acid dehydroxylase	Cholic and chenodeoxycholic acids
Amino acid deaminase	Tyrosine and other amino acids

BACTERIAL XENOBIOTIC METABOLISING ENZYMES

A list of the major bacterial enzymatic reactions leading to alterations in the toxicity of substrates is given in Table 2. Some of the reactions are considered in more detail below.

ß-Glycosidases

Plants produce a wide variety of secondary metabolites including azoxy, anthraquinone, diterpenoid and flavonoid structures which are usually stored as glycosides (*Brown*, 1988). Their presence, often in large quantities, in edible fruits and vegetables and in beverages, such as tea and wine, results in significant human intake (*Hertog* et al., 1993). Glycosides are poorly absorbed in the small intestine and pass into the colon, where the action of bacterial ß-glycosidases cleaves the sugar moiety releasing aglycones, which exhibit a wide range of biological activities. For example, methylazoxymethanol (MAM), the hydrolysis product of cycasin, is carcinogenic (*Laqueur* and *Spatz*, 1968) and many of the flavonoid, anthraquinone and diterpenoid aglycones exhibit mutagenic activity in *in vitro* assays, although apart from MAM, their carcinogenic effects are debatable (*Brown*, 1988).

Assessment of the toxicological significance of glycoside hydrolysis by intestinal microflora is further complicated by reports of anti-carcinogenic and anti-mutagenic effects of flavonoid aglycones against a wide variety of carcinogens including benzo(a)pyrene (*Wattenberg* and *Leong*, 1970), 4-dimethylaminoazobenzene (*Nagase* et al., 1964) and the cooked food mutagens IQ, MeIQ and MeIQx (*Alldrick* et al., 1986).

ß-Glucuronidase

Many xenobiotics and endogenously-produced compounds are metabolised in the liver and conjugated to glucuronic acid before being excreted into the small intestine in bile (*Smith*, 1966). In the colon, hydrolysis of the glucuronide linkage by bacterial ß-glucuronidase can release the parent compound, or its hepatic metabolite. Reabsorption of the compound can result in its enterohepatic circulation with concomitant potentiation of its pharmacological or toxicological effects. Many carcinogens including polycyclic aromatic hydrocarbons, heterocyclic amines (e.g. IQ) can be conjugated and secreted into the intestine where ß-glucuronidase action may release the parent carcinogen in the colon. For example, benzo(a)pyrene (BP), a contaminant of the human diet, undergoes activation and conjugation in the liver. Gut bacteria have been shown to release reactive metabolites from biliary conjugates which covalently to DNA and are genotoxic (*Renwick* and *Drasar*, 1976; *Chipman* et al., 1983).

Nitrate reductase

Nitrate, ingested with the diet and drinking water, is readily converted by gut bacteria to its more reactive and toxic reduction product, nitrite which can react with nitrogenous compounds in the body to produce N-nitroso compounds many of which are highly carcinogenic (*Rowland*, 1988). Using germ-free rats, we have demonstrated the importance of the gut microflora for this nitrosation reaction (*Massey* et al., 1988) and recently have shown that the reaction can occur in man by measuring N-nitroso compounds in faeces (*Rowland* et al., 1991).

Azoreductase

A number of dyes used in food, cosmetics and for textiles and leather are based on azo compounds which are reduced in the gut by the intestinal flora to produce, ultimately, amines. In some cases the reduction products are toxic. For example, workers exposed to Direct Black 38, a dye used in the leather and textile industry, have an elevated risk of bladder cancer which has been attributed to the reduction of the dye by the gut microflora to benzidine, a known human bladder carcinogen (*Powell*, 1979; *Cerniglia* et al., 1982).

Nitroreductase

Heterocyclic and aromatic nitro compounds are important chemical intermediates (*Hartter*, 1984), are used as antibiotics and radiosensitising drugs and are ubiquitous environmental pollutants resulting from combustion of fossil fuels. Many of these compounds possess toxic, mutagenic and carcinogenic activity and so may contribute to the environmental cancer risk in man (*Rosenkranz* and *Mermelstein*, 1982; *Busby* et al., 1985). Reduction of the nitro group is usually required for the pharmaceutical and toxicological activity of these compounds to be expressed (*Lindmark* and *Muller*, 1976; *Reddy* et al., 1976). Although reduction of the nitro group can be effected by both mammalian and bacterial reductases nitroreduction by the gut microflora appears to play a more important role than hepatic enzymes particularly in the cases of nitrobenzenes (*Reddy* et al., 1976), dintrotoluenes (*Doolittle* et al., 1983; *Mirsalis* et al., 1982) and nitrated polycyclic hydrocarbons, such as 6-nitrochrysene (*Cerniglia* et al., 1982; *Rickert*, 1988).

Bacterial metabolism of 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (IQ)

IQ is one of several heterocyclic amine compounds which are produced in small amounts when meat and fish are grilled or fried (*Felton* et al., 1986).

Table 3: Possible mechanisms by which probiotics exert beneficial effects via gut flora metabolism

1. Probiotic displaces or dilutes indigenous gut flora organisms with high enzymic activities, thus suppressing, overall, reactions which result in generation of toxic or carcinogenic metabolites.
2. Probiotic generates conditions in the gut which alters the rate of bacterial activation of ingested chemicals e.g. lowering of pH (affects ammonia production, bile acid metabolism).
3. Probiotic stimulates reactions which lead to the production of potentially beneficial products in the gut e.g. anti-carcinogenic flavonoids.

These compounds are normally mutagenic in *Salmonella typhimurium* only after activation by hepatic cytochrome P450-dependent mixed function oxidases (*Yamazoe* et al., 1983; *Alldrick* et al., 1986). IQ is also carcinogenic in rodent bioassays and induces tumours at various sites including the large intestine, suggesting that it may play a role in the aetiology of colon cancer in man (*Ohgaki* et al., 1986).

Incubation of IQ with a suspension of human faeces yields the 7 keto derivative, 2-amino-3,6-dihydro-3-methyl-7*H*-imidazo[4,5-*f*]quinoline-7-one (7-OHIQ) (*Bashir* et al., 1987) and the reaction has been shown not to occur in gut contents obtained from germ-free rats (*Rumney* et al., 1993). Unlike IQ, the bacterial metabolite is a direct-acting mutagen in *Salmonella typhimurium* (*Carman* et al., 1988, *Rumney* et al., 1993). Thus there is strong evidence for the bacterial formation in the human gut of a directly genotoxic derivative of a dietary carcinogen.

EFFECT OF PROBIOTICS AND OLIGOSACCHARIDES ON XENOBIOTIC METABOLISM BY THE GUT MICROFLORA

One of the most important ways in which a probiotic organism may exert a beneficial effect on its host is to modify the reactions (described above and in Table 2) leading to the generation of potentially toxic products in the gut. Such a beneficial effect could be achieved in theory in a number of ways (Table 3).

The colonic flora of man and other mammals is a highly complex ecosystem comprising over 400 different species. Some limited information on the ability of some of these species to catalyse metabolic reactions *in vitro* has been reported and these studies indicate a wide range of metabolic capacity within and between the various groups (*Cole* et al., 1985; *Saito* and *Rowland*, 1992). In general, however, species of *Bifidobacterium* and *Lactobacillus*, which are commonly used as probiotics, have low activities of xenobiotic metabolising enzymes such as azoreductase, nitroreductase, nitrate reductase and ß-glucuronidase, by comparison to other major anaerobes in the gut such as bacteroides, eubacteria and clostridia (Table 4). Conversely, they have high levels of glucosidase activity which, as described above, may result in the generation in the gut of flavonoid aglycones with genotoxic and anti-carcinogenic properties.

The number of organisms that has been investigated in this way is limited,

Table 4: Enzyme activities of intestinal organisms *in vitro*

Species	Enzyme activities (µmol/h/10^{10}cells)			
	Azo	GN	GS	NR
Bifidobacterium				
longum	ND	0.001	0.20	NE
adolescentis	ND	ND	0.65	NE
breve	ND	ND	3.67	NE
infantis	ND	ND	2.73	NE
bifidum	ND	ND	0.11	NE
Bacteroides				
fragilis	0.01	0.007	0.514	NE
vulgatus	0.03	0.012	0.064	NE
thetaiotaomicron	0.02	ND	0.815	NE
uniformis	0.09	0.006	1.860	NE
sp IY37	NE	0.680	5.380	0.01
Clostridium				
perfringens	0.343	0.02	0.84	NE
paraputrificum	7.530	0.03	8.20	NE
innocuum	0.230	0.04	0.55	NE
Eubacterium				
aerofaciens	0.348	0.012	0.166	NE
lentum	ND	ND	0.004	NE
Streptococcus sp.	NE	0.04	0.03	0.0004
Lactobacillus				
salivarius	NE	ND	0.10	ND

After *Cole* et al. (1985), and *Saito* & *Rowland* (unpublished observations, 1991).

NE = Not estimated
ND = Not detected
Enzymes Key : Azo = azoreductase; N = ß-glucuronidase; GS = ß-glucosidase; NR = nitroreductase.

hence the abilities of the many component species of the microflora to metabolise nutrients and foreign compounds are not known in any detail. A further complication is that although a species may express an enzyme activity when cultured *in vitro*, the same species may behave very differently when it colonises the gut of an animal. This phenomenon was demonstrated by comparing enzymatic activity of microflora strains *in vitro* with the activity *in vivo* by using gnotobiotic animals monoassociated with specific gut microorganisms (*Cole* et al., 1985).

Despite these caveats, the evidence suggests that increasing the numbers of lactic acid bacteria in the gut could modify, beneficially, the levels of xenobiotic metabolising enzymes.

Two main strategies have been employed in attempts to increase the level of potentially beneficial organisms in the gut. The direct approach is to supply live preparations of probiotics which can be added to the diet. The indirect approach is to use non-digestible, carbohydrate food supplements (oligosac-

charides) which support and stimulate the growth of lactic acid bacteria in the colon.

Effects of probiotic consumption on gut bacterial metabolism

The effect of administration of probiotic organisms on bacterial enzymes of toxicological importance has been addressed in a number of papers.

In a rat study, supplementation of a high meat diet (72% beef) with *L. acidophilus* (to provide 10^9-10^{10} organisms/day) significantly decreased by 40-50% the activity of faecal ß-glucuronidase and nitroreductase. It is noteworthy that the modulating effect of the lactobacillus strain was dependent on the type of diet fed - no significant effect on enzyme activities were seen when the rats were fed grain-based diet (*Goldin* and *Gorbach*, 1977).

These studies have been extended to humans who were given milk supplemented with 10^9 viable lactobacilli per day (*Goldin* and *Gorbach*, 1984). Prior to lactobacillus feeding, faecal ß-glucuronidase activity ranged between 1.74 and 2.14 units/mg faecal protein. After ingestion of the lactobacilli for 30 days, the activity of ß-glucuronidase declined in all 21 subjects (mean value of 1.12 units/mg protein). Enzyme activities returned to the baseline value 10 days after lactobacillus consumption ceased. In the case of nitroreductase and azoreductase, decreases in enzyme activity during lactobacillus exposure of over 75% were reported and control values were not restored until 30 days after lactobacillus supplementation ceased. The fact that the changes in enzyme activity were not sustained when the ingestion of lactobacilli ceased indicates that the intestinal tract was not permanently colonised.

In an analogous experiment to those of *Goldin* and *Gorbach*, *Cole* and colleagues (1989) investigated the effect of *L. acidophilus* administration on bacterial metabolic activities in germ-free rats colonised with a human faecal microflora. A significant reduction in ß-glucosidase and ß-glucuronidase activities was observed when lactobacilli were given for 3 days with the effects persisting for 7 days after dosing ceased.

Effect of probiotics on *in vivo* metabolism of xenobiotics

The changes in enzyme activities seen in the animals and humans treated with lactobacilli would, in theory, be expected to result in changes in rates of metabolism of their substrates *in vivo*, although only if the enzymes catalysed the rate limiting step in their metabolism. It is important, therefore, to test that this is in fact the case and that the changes in enzyme activities observed do result in corresponding changes *in vivo* metabolism of potentially toxic chemicals. *Goldin* and *Gorbach* (1984a) have investigated this by feeding to rats aromatic nitro compounds (nitrofluorene, nitronaphthalene), an azo dye and a glucuronide (2-naphthylamine-N-D-glucuronide) and monitoring the production of the reaction products, i.e. free amines, in faeces. It should be noted that the rats were fed a high meat (72%, w/w) diet to increase enzyme activities and so maximise the effect of the lactobacilli on the enzymes (see above).

The reduction in activity of nitroreductase, azoreductase and ß-glucuronidase in the rats given oral lactobacilli supplements, was matched by a decrease, about 50% by comparison to controls, in the excretion of free amine products. These results show that administration to laboratory animals of lactobacilli can decrease the production of toxic and carcinogenic amines (e.g. 2-naphthylamine, a human bladder carcinogen) from ingested substrates.

The effect of ingestion by human subjects of *Lactobacillus casei* on urinary excretion of potentially toxic amino acid metabolites was studied by *Tohyama* and colleagues (1981). The urinary concentration of indican (from tryptophan) and p-cresol (from tyrosine) was significantly decreased by feeding 10^{10} organisms/day for 5 weeks with mean reductions of 29 and 43% respectively.

Effect of probiotics on tumorigenesis

The influence of *B. longum* on induction of colon and mammary tumours by the cooked food carcinogen IQ has recently been investigated by *Reddy* and co-workers (1993). Rats were fed, for 58 weeks, a high fat diet containing 125 ppm IQ with or without a dietary supplement (0.5%) of freeze-dried *B. longum*. In male rats, significant decreases were reported in both the incidence of colon tumours (43% incidence in rats fed IQ alone, no tumours in those fed IQ plus *B. longum*) and the number of tumours per animal. In female rats, the number of mammary tumours per animal, but not tumour incidence, was significantly decreased. One of the postulated mechanisms for the anticarcinogenic effect was inhibition by the bifidobacteria of the deconjugation of biliary conjugates of IQ in the colon, which would have the effect of reducing the release of free IQ and/or its genotoxic metabolites in the colon (see section on ß-glucuronidase above)

Effect of milk (fermented and non-fermented) and yoghurt on metabolism by gut microflora

Ingestion by elderly (>65 yr.) volunteers of non-fermented milk containing *L. acidophilus* was associated with minor and inconsistent changes in activity of ß-glucuronidase and ß-glucosidase in faeces (*Ayebo* et al., 1980). The lack of effects were possibly due to the low level of lactobacilli (about 2 x 10^6 organisms per ml) in the milk which resulted in only minor increases in the faecal lactobacillus count.

In a study by *Marteau* and co-workers (1990), volunteers consumed a fermented milk product containing higher numbers of lactic acid bacteria : *L. acidophilus* (10^7/g), *Bifidobacterium bifidum* (10^8/g) and *Streptococcus lactis* and *S. cremoris* (both at 10^8/g). Although azoreductase and ß-glucuronidase activities did not change in response to consumption of the fermented milk, nitroreductase activity was decreased by 38% and remained depressed for at least 3 weeks. In contrast, ß-glucosidase increased after ingestion of the fermented milk - a change attributable to the high activity of this enzyme in *B. bifidum*.

In the various studies, described above, performed using either lactobacilli or milk products containing lactobacilli, the most consistent finding is a decrease in nitroreductase activity in faeces. The inconsistency of the other changes in metabolism may be due to differences in the types of probiotic fed or their concentrations in the product.

Effect of oligosaccharides on gut bacterial numbers and metabolism

A number of oligosaccharides (short chain-length sugars, usually less than 20 monomers in the chain) have been isolated which are not substrates for mammalian hydrolytic enzymes and so pass undegraded into the colon where they may be fermented by colonic bacteria. These oligosaccharides are usually only fermented by a limited range of microorganisms and so in theory can selectively stimulate the growth of chosen organisms. They are sometimes co-administered with a specific organism (usually a *Bifidobacterium* species) in

order encourage its multiplication in the gut and hence potentiate the beneficial, "probiotic" effects of the organism. Although not strictly probiotics, these indigestible sugars have been included in this review since they possess many of the properties of probiotics.

The main oligosaccharides that have been studied for beneficial effects on the consumer are :

a) Transgalactosylated oligosaccharides (TOS; *Tanaka* et al., 1983). TOS, a mixture of tri-, tetra-, penta- and hexa-saccharides of galactose and glucose, was utilised by all *Bifidobacterium* species tested and by some lactobacilli, bacteroides, streptococci and enterobacteria (*Tanaka* et al., 1983).
b) Soybean oligosaccharides extract (SOE; *Hayakawa* et al., 1990). SOE comprises a mixture of sucrose (44%), stachyose (23%), raffinose (7%) and monosaccharides and is obtained from defatted soybean whey. The extract can be further refined (SOR) to increase the stachyose and raffinose content to 71 and 20% respectively. The efficiency of utilisation of SOR by intestinal bacteria *in vitro* was greatest with *Bifidobacterium* species although other genera including *Lactobacillus* and *Bacteroides* were capable of fermenting the sugar.
c) Fructo-oligosaccharides (*Hidaka* et al., 1986; *Rumessen* et al., 1990). A commercial preparation of oligosaccharides ("Neosugar"), a mixture of tri-, tetra- and penta-saccharides of glucose and fructose, is utilised by most bifidobacteria, bacteroides and some streptococci, lactobacilli and enterobacteria, but not *E. coli* (*Hidaka* et al., 1986). Another oligofructose, derived from inulin which is found in garlic, chicory, artichoke and onion, behaves in a similar manner towards gut bacteria, being fermented mainly by bifidobacteria (*Wang* and *Gibson*, 1993).

The effect of ingestion of TOS (3 or 10g/d), *B. breve*, or both, on faecal bacterial counts and faecal ammonia has been investigated in human volunteers (*Tanaka* et al., 1983). In general, significant effects were seen only during periods when both TOS and *B. breve* were ingested simultaneously. For example, during these periods, the viable counts of bacteroides and enterobacteria in faeces declined and faecal ammonia concentration decreased markedly in 4 out of the 5 volunteers. Ingestion of TOS alone even at 10g/d , gave inconsistent effects on these parameters although the study may have been limited by the small number of participants. A study in human-flora-associated (HFA) rats has extended the above human study to encompass bacterial metabolic activities as well as bacteriological analyses (*Rowland* and *Tanaka*, 1993).

The HFA rats were fed, for 4 weeks, a purified diet with or without TOS (5% w/w). Caecal concentrations of bifidobacteria and lactobacilli were significantly increased in TOS-fed rats, whilst enterobacteria were decreased and bacteroides were unaltered. Bacterial ß-glucuronidase and nitrate reductase activities, pH and conversion of the dietary carcinogen IQ to its directly genotoxic 7-hydroxy derivative, were significantly reduced in caecal contents of TOS-fed rats. Bacterial ß-glucosidase activity was increased presumably as a consequence of the elevated numbers of bifidobacteria which have high levels of this enzyme (Table 4; *Saito* et al., 1992). The results of this study indicate that TOS-induced changes in microflora may be potentially beneficial due to decreased bacterial activities associated with generation of toxic, genotoxic and carcinogenic products in the gut.

Ingestion of Neosugar (8g/d for 14d)

by elderly patients led to a slight increase in total bacterial count in faeces and about a 10-fold increase in bifidobacteria (*Hidaka* et al., 1986). However, the increase in bifidobacteria was seen only in those individuals who originally had low faecal counts ($<10^8$/g) of the organisms. Similar inter-individual differences in response were seen in gut bacterial metabolism when Neosugar was administered for 2 months at a dose rate of 8g/d. An increase in bifidobacteria was accompanied by a rise in short-chain fatty acid (SCFA) concentration in faeces and a decrease in p-cresol and indole. In the same person, however, faecal ammonia was increased nearly two-fold. Again, changes in these metabolic profiles were not seen in a person whose bifidobacteria count was initially high. (*Hidaka* et al., 1986). These studies need to be interpreted with extreme caution since only two individuals were studied.

Further experiments were performed in rats fed a purified diet containing tyrosine and tryptophan. Incorporation of Neosugar into the diet at 0.4-10% appeared to reduce p-cresol concentration in faeces especially at the highest dose of the oligosaccharide (*Hidaka* et al., 1986). As might be expected, high dietary concentrations of Neosugar (10-20%) in the diet of rats also markedly increased SCFA concentration in faeces (*Tokunaga* et al., 1986). The total daily excretion of neutral steroids was also increased although an increase in bile acid excretion was seen only at the higher (20%) dose, which is outside the normal human intake level. The changes in faecal neutral steroid excretion in rats were not reflected in serum cholesterol concentrations suggesting that Neosugar stimulates cholesterol synthesis rather than just increasing faecal excretion (*Tokunaga* et al., 1986).

A study of soy bean oligosaccharides (SOE) (10 g/day) in human volunteers demonstrated that the viable count of bifidobacteria in faeces increased slightly but significantly during the period of ingestion of oligosaccharide preparation (*Hayakawa* et al., 1990). Intake of *B. breve* with SOE appeared to have little additional effect on bifidobacteria numbers in faeces. Faecal pH and amino acid degradation products in faeces (indole, p-cresol and phenol) were also determined, but no consistent significant differences were observed between the various dietary periods.

The refined soybean oligosaccharide SOR has been studied in an *in vitro* continuous flow culture system which models the human colonic microflora (*Bearne* et al., 1990; *Saito* et al., 1992). Numbers of bifidobacteria were determined and various bacterial enzyme activities assayed before and after SOR was incorporated into the growth medium at a concentration of 0.1% (w/v). After addition of SOR, there was a 5-fold increase in concentration of bifidobacteria in the culture, which mirrors the changes seen in human volunteers (*Hayakawa* et al., 1990). The activity of some bacterial enzymes was also altered during the period of SOE supplementation. Azoreductase activity decreased significantly ($p<0.05$) by 40% and ß-glucuronidase and ß-glucosidase activities also decreased (by 38 and 32%, respectively), although the results were not significant. In a subsequent study, HFA rats fed 3% refined soy-bean oligosaccharides in the diet showed decreased excretion of N-nitroso compounds in faeces suggesting that soy bean oligosaccharides may decrease exposure of the colon to these potentially carcinogenic substances.

CONCLUSIONS

There is good evidence that certain strains of lactobacilli can modify intestinal bacterial metabolism of foreign compounds. In some cases, the biological and toxicological significance of the changes seen has been established and indicates that ingestion of such probiotic organisms may have beneficial effects in humans. Other probiotic organisms such as bifidobacteria have been studied less extensively with regard to their influence on intestinal bacterial metabolism. Their effects on the bacterial flora of the gut have been investigated, but such studies, although valuable in establishing that a treatment can modify the flora, give little information on the biological consequences of for the host animal. Further metabolic and toxicological studies are urgently needed therefore in this area. The use of indigestible oligosaccharides to stimulate growth of lactic acid bacteria in the intestine holds great promise and presumably these food supplements will continue to be refined in terms of the specificity of their effects. Again, metabolic and toxicological studies will be necessary for assessing their beneficial consequences for the consumer.

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PROBIOTICS: PROSPECTS FOR USE IN *CLOSTRIDIUM DIFFICILE*-ASSOCIATED INTESTINAL DISEASE

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INTRODUCTION

Clostridium difficile is an etiologic agent of a spectrum of gastrointestinal diseases which range in severity from uncomplicated diarrhoea to fulminating pseudomembranous colitis. Despite the availability of effective pharmacotherapy, *C. difficile* colitis remains a serious condition, especially in elderly and debilitated patients. This paper will review the evidence that the normal intestinal flora is an important barrier to *C. difficile* intestinal colonisation and will examine some of the parameters possibly involved in colonisation resistance to *C. difficile* infection. The role of probiotics in the treatment and prevention of *C. difficile*-mediated intestinal disease will also be discussed.

CLOSTRIDIUM DIFFICILE-ASSOCIATED INTESTINAL DISEASE

Clostridium difficile-induced intestinal disease in humans is a health problem of significant clinical importance. Toxigenic *C. difficile* is the major cause of antibiotic-induced pseudomembranous colitis (PMC), a severe and life-threatening intestinal disease, and is the cause of approximately one-third of cases of antimicrobial agent-associated non-specific colitis and diarrhoea without colitis (*George*, 1988; *Bartlett*, 1992). *C. difficile*-associated intestinal disease results from antibiotic suppression of the indigenous intestinal flora with resultant proliferation of *C. difficile*. All major classes of antimicrobial agents have been reported to induce *C. difficile*-associated intestinal disease in humans (*Trnka* and *LaMont*, 1984). The major offending antibiotics are ampicillin, cephalosporins, and clindamycin, given either singly or in combination with other antibiotics. *C. difficile*-associated intestinal disease can occur during antimicrobial therapy or begin weeks after discontinuation of therapy and may persist for months. Since its discovery as the cause of PMC in 1978, *C. difficile* has emerged as the major identifiable infectious cause of nosocomial diarrhoea in the United States, infecting 15% to 25% of adult hospitalised patients (*DuPont* and *Ribner*, 1992; *Bartlett*, 1990; *McFarland* et al., 1989). Nosocomial *C. difficile* infection causes significant morbidity and is associated with increased hospital costs and lengths of stay (*Kofsky* et al., 1991).

The mechanisms by which *C. difficile* causes intestinal mucosal injury and death are not entirely understood. However, the pathogenicity of this microorganism is dependent, at least in part, upon two biochemically and immunochemically distinct toxins produced during replication of *C. difficile* in the intestine. These toxins are referred to as

toxin A (enterotoxin) and toxin B (cytotoxin). There is considerable evidence implicating both toxins in the development of *C. difficile*-induced disease (*Donta*, 1988; *Lyerly* and *Wilkins*, 1988). Toxin A is thought to be responsible for most of the diarrhoea and damage to the colonic mucosa seen in PMC (*Mitchell* et al., 1986; *Triadafilopoulos* et al., 1987). Although purified toxin B has no effect when administered alone into the intestinal tract, it is a potent cytotoxin for most mammalian fibroblast cell lines *in vitro* (*Lyerly* et al., 1985). It appears that toxin A binds to and causes lesions on the intestinal epithelium. Through the intestinal lesions, toxins A and B act on the underlying tissue and structures. If *C. difficile*-induced intestinal disease is left untreated, these toxins can result in severe systemic effects and death.

Since the discovery that *C. difficile* is the primary etiologic agent of antimicrobial agent-induced PMC, therapy directed at *C. difficile* has led to generally excellent results (*Finegold* and *George*, 1988). Many or most patients with *C. difficile*-related intestinal disease run a self-limited course so that simply discontinuing the inciting agent or agents usually results in clearing of diarrhoea within a few days (*Triadafilopoulos* et al., 1987). No further specific therapy will be required in a substantial number of patients, particularly those with mild diarrhoea and without signs and symptoms of colitis. If therapy for the initial infection is still required, switching to another agent that has less impact on the normal faecal flora, and that is less likely to lead to *C. difficile*-induced disease, may allow recovery from the gastrointestinal complication of earlier therapy.

In severe cases of *C. difficile*-associated intestinal disease or in those that do not respond to the cessation of the inciting antibiotic, specific antimicrobial therapy against *C. difficile* has proven useful. The most widely employed and most effective treatment of *C. difficile*-associated diarrhoea or colitis is the use of orally administered vancomycin (*Triadafilopoulos* et al., 1987; *Keighley* et al., 1978; *Fekety* et al., 1981). Symptomatic improvement is usually evident within 24 to 48 hours of initiating therapy, and complete resolution of diarrhoea and colitis occurs in the majority of patients by the end of a 10 day treatment period. Additional antimicrobial agents used in the treatment of *C. difficile*-associated colitis are bacitracin and metronidazole (*Triadafilopoulos* et al., 1987; *Young* et al., 1985; *Dudley* et al., 1986; *Teasley* et al., 1983; *Young* et al., 1985). Clinical trials have indicated that these two agents are comparable to vancomycin in efficacy. However, occasional metronidazole and bacitracin resistant strains of *C. difficile* have been reported (*Bartlett*, 1985; *Saginur* et al., 1980).

Despite an excellent initial response to therapy, the discontinuation of therapy is followed by a relapse of *C. difficile*-associated intestinal disease in approximately 20% of patients irrespective of which antimicrobial agent is used (i.e., metronidazole, vancomycin, bacitracin) (*Bartlett* et al., 1980a; *Walters* et al., 1981; *George* et al., 1980; *Teasley* et al., 1983; *Fekety* et al., 1989). The signs and symptoms of relapse are similar to the initial attack. Once patients experience a recurrence of *C. difficile* disease, they are more likely to have subsequent recurring episodes of the disease (*Bartlett*, 1983). It is thought that re-infection occurs when the concentration of antibiotic has dropped below that to which *C. difficile* is sensitive and before the normal flora has had a chance to regain its equilibrium (*Young* and *McDonald*, 1986). It is thought that *C. difficile* survives in the intestinal tract during therapy in the form of antibiotic-

resistant spores since symptomatic relapse frequently involves re-infection with the same strain of *C. difficile* that caused the initial infection. It is also possible that some relapse cases may actually represent exogenous re-infection acquired in the hospital setting since it is known that the organism persists in the environment for extended periods (*Johnson* et al., 1989). Presently there is no reliable way to predict the likelihood of relapse in any one individual. Relapses occur whether the vancomycin has been given in high dose or low dose, for long periods or short periods. Furthermore, persistence of the toxins or the organism at the completion of therapy is not predictive of the likelihood of relapse. The relapses are often cured by a second course of antimicrobial therapy (*Bartlett* et al., 1980b). However, multiple relapses, involving the reappearance of the organism with its cytotoxin in the stool, can occur. Management of the patient with multiple relapses can be very difficult since no single therapeutic measure is uniformly effective in preventing disease relapse. Traditional approaches to treating patients with recurring *C. difficile* colitis have included repeated courses of antibiotics, addition of resins such as cholestyramine and colestipol, and longer tapering doses or pulse doses of vancomycin (*Bartlett*, 1983; *Tedesco* et al., 1985).

The variety of therapies tried in treating patients with relapse of *C. difficile*-associated intestinal disease attest to the fact that no single therapeutic measure is uniformly effective in preventing disease relapse. Optimal therapy of initial *C. difficile* disease as well as relapse should take into account the important protective role of the normal bowel flora. This has stimulated various groups of researchers to try to identify components of the normal bowel flora that are involved in excluding *C. difficile* from the intestinal tract.

IMPORTANCE OF NORMAL FLORA IN COLONISATION RESISTANCE TO *CLOSTRIDIUM DIFFICILE*

The mechanisms which permit *C. difficile* overgrowth in the intestinal tracts of humans are unclear. The normal flora of the gastrointestinal tract provides an important protective barrier against infection by enteric pathogens and there is general agreement that this is particularly important for protection against gastrointestinal colonisation by *C. difficile*. The ability of the normal flora of the lower gastrointestinal tract to maintain an ecologic balance and prevent colonisation by pathogens and exogenous microorganisms is known as "colonisation resistance" (*van der Waaij* et al., 1971). *C. difficile* intestinal overgrowth is precipitated by factors which disturb the ecology of the gastrointestinal tract. The majority of cases of *C. difficile*-mediated intestinal disease are a result of antimicrobial agents altering the composition of the normal intestinal flora so as to permit colonisation and/or proliferation by *C. difficile* as well as toxin elaboration by the organism. Individuals who contract *C. difficile*-associated intestinal disease may either be a carrier of low numbers of this microorganism at the time of antibiotic exposure or acquire the microbe from an environmental source.

Several investigators have presented *in vitro* and *in vivo* experimental evidence to show that the normal intestinal flora acts as a natural barrier that effectively interferes with the establishment of *C. difficile*. Table 1 outlines the experimental approaches which have been

Table 1: Experimental approaches to examine colonisation resistance against *Clostridium difficile*

In vitro experiments	*In vivo* experiments
Antagonism between individual isolates on agar media	Antibiotic-treated animals
Continuous flow culture	Gnotobiotic animals
Batch culture	Infant animals

used to study colonisation resistance to *C. difficile*. The results of some of these studies are summarised below.

In Vitro Studies of Colonisation Resistance

Antagonism Between Individual Isolates on Agar Media

A number of faecal bacteria have been identified as being antagonistic to the growth of *C. difficile* (Table 2). *Rolfe* et al. (1981) examined representative faecal bacteria from 23 anaerobic and aerobic genera for antagonism against *C. difficile in vitro*. Strains of bacteria in six of the genera inhibited the multiplication of *C. difficile*, with lactobacilli and group D enterococci displaying the greatest antagonistic activity. *Malamous-Ladas* and *Tabaqchali* (1982) also demonstrated *in vitro* antagonism between faecal streptococci and *C. difficile*. *Barclay* and *Borriello* (1982) have isolated strains of *C. beijernickii* from human faeces which exhibit almost total specific antagonism for *C. difficile*. These studies demonstrate the occurrence of inhibitory interactions between bacterial components of the normal intestinal flora and *C. difficile*. However, these types of studies are obviously limited in that the artificial conditions used relate poorly to those found *in vivo*.

Continuous Flow Culture

In vitro studies of interactions between *C. difficile* and other bacteria have also been conducted in continuous flow cultures in a chemostat. *Wilson* and *Freter* (1986) attempted to establish a complete hamster caecal flora in continuous flow culture by seeding a hamster pellet extract medium with caecal contents from a healthy hamster. Continuous flow cultures were colonised first with *C. difficile* and then the caecal

Table 2: Organisms antagonistic on agar media to growth of *Clostridium difficile*

Aerobic organisms	Anaerobic organisms
*Pseudomonas aeruginosa**	*Clostridium beijerinckii****
*Staphylococcus aureus**	*Bacteroides* spp.*
Group D Enterococci*	*Bifidobacterium adolescentis**
*Streptococcus faecalis***	*Bifidobacterium infantis**
*Streptococcus faecium***	*Bifidobacterium longum**
*Streptococcus mitis**	*Lactobacillus* spp.*
Streptococcus spp.**	

* *Rolfe* et al., 1981.
** *Malamous-Ladas* and *Tabaqchali*, 1982.
*** *Borriello* and *Barclay*, 1982.

flora of hamsters. In these experiments, the numbers of *C. difficile* present in the continuous flow culture were reduced from a mean $\log_{10}$ CFU/ml of 8.3 to a mean of 2.7. However, in studies were 150 bacterial isolates from the established caecal flora were used, *C. difficile* levels were reduced by a factor of only 2 logs (*Wilson* and *Freter*, 1986). These investigators concluded that synthetic floras must themselves be very complex to simulate the functions of the natural flora and that further work to develop a synthetic microflora to suppress *C. difficile* must be based on a knowledge of the control mechanisms normally active against this pathogen.

Yamamoto et al. (1989) demonstrated that in a mixed anaerobic continuous flow culture containing *Streptococcus parvulus* and *C. difficile*, the cytotoxin levels were significantly reduced compared to cultures containing *C. difficile* alone. However, there were no differences between growth of *C. difficile* in mixed and single cultures. Additional experiments indicated that the suppressive effect of *S. parvulus* on the cytotoxin activity of *C. difficile* was not due to the inactivation of the extracellular cytotoxin, but due to inhibition of the intracellular synthesis of cytotoxin. The precise mechanism of the inhibitory activity was not determined by the investigators.

Batch Culture

An *in vitro* model for studying colonisation resistance to *C. difficile* has been developed by *Borriello* and *Barclay* (1986) based on monitoring the growth of the organism and toxin production in faecal emulsions prepared from the faeces of different patient groups and healthy subjects of different ages. In these experiments, faeces were homogenised in distilled water and seeded with a toxigenic strain of *C. difficile*. Growth of *C. difficile* was inhibited when in faecal emulsions derived from the stools of healthy adults. On the other hand, faecal emulsions sterilised by either filtration or autoclaving permitted *C. difficile* growth and cytotoxin production showing the importance of viable bacteria (*Borriello* and *Barclay*, 1986). Faecal emulsions derived from stools of healthy subjects of different age groups demonstrated that those from geriatrics, children, and bottle fed infants were less inhibitory that those from healthy adults (*Borriello* and *Barclay*, 1986). Interestingly, these groups have a higher incidence of *C. difficile* intestinal colonisation than healthy adults. The faecal emulsions derived from patients with antibiotic-associated diarrhoea fell into two main groups: those that were inhibitory and those that were not. The investigators could not confirm that the subjects yielding non-inhibitory emulsions were susceptible to infection with *C. difficile*. However, they have recently shown that this *in vitro* batch culture model is predictive of outcome of infection in antibiotic pretreated hamsters (*Borriello* et al., 1988). The investigators speculated that it may be possible to identify those patients at risk of developing *C. difficile*-mediated intestinal disease using this *in vitro* system. This model is also being used to help identify the strains of bacteria responsible for colonisation resistance against *C. difficile*. For example, the removal of facultative Gram-negative bacteria from faecal emulsions has no effect on colonisation resistance whereas removal of anaerobic bacteria does.

In Vivo Studies of Colonisation Resistance

Antibiotic Treated Animals

A number of studies have been undertaken in hamsters and mice to test the hypothesis that intestinal flora compo-

Table 3: Reconstitution of colonisation resistance using complete flora from a donor of the same or a different species

Host	Donor flora	Reference
Antibiotic-Treated Hamster	Hamster	Wilson, Silva and Fekety, 1981 Larson and Welch, 1993
Antibiotic-Treated Hamster	Human	Larson and Welch, 1993
Germfree Mouse	Hamster	Wilson et al., 1986 Jin et al., 1984 Wilson and Freter, 1986 Boureau et al., 1990
Germfree Mouse	Mouse	Wilson et al., 1986 Itoh et al., 1987
Germfree Mouse	Hare	Ducluzeau et al., 1981
Germfree Mouse	Human	Raibaud et al., 1980
Newborn Hare	Hare	Ducluzeau et al., 1981

nents that normally suppress *C. difficile* are eliminated by antibiotic administration, allowing the pathogen to attain unusually high population levels (Table 3). These experiments have uniformly shown that the intestinal tracts of antibiotic-treated animals are readily colonised with *C. difficile*, whereas non-antibiotic treated adult animals harbouring a conventional flora are resistant to colonisation (*Wilson* et al., 1985; *Larson* et al., 1980; *Toshniwal* et al., 1981). For example, the golden Syrian hamster has been the most widely employed animal model of antibiotic associated colitis caused by toxigenic *C. difficile*. Non-antibiotic treated adult hamsters rarely harbour *C. difficile* and even large numbers of *C. difficile* administered intracaecally into normal hamsters is eliminated by 24 hours (*Wilson* et al., 1985; *Larson* et al., 1980; *Toshniwal* et al., 1981; *Larson* and *Borriello*, 1990). On the other hand, *C. difficile* rapidly attains a large population size when introduced into antibiotic treated hamsters and a fatal ileo-caecitis rapidly ensues (*Wilson* et al., 1985; *Larson* and *Borriello*, 1990). Orogastric and rectal administration of faecal homogenates obtained from normal hamsters or human volunteers decreases the number of viable *C. difficile* and prevents caecitis in antibiotic challenged hamsters (*Larson* and *Welch*, 1993; *Wilson* et al., 1981). The protective effect of these homogenates is destroyed by heating to 100°C for 20 min or by filtering them through a 0.22 µm membrane filter indicating the importance of viable bacteria (*Wilson* et al., 1981). The protective effects of homogenates were also lost with exposure to clindamycin but not with exposure to vancomycin or gentamicin suggesting that only certain bacterial components of the homogenates are involved in preventing the establishment of *C. difficile* in the intestine (*Wilson* et al., 1981). Attempts to determine which antibiotic-induced changes are important in allowing *C. difficile* to colonise in numbers large enough to cause disease have not been successful because of the massive

Table 4: Asymptomatic intestinal colonisation by *Clostridium difficile* in infants

Number of infants positive for *C. difficile* (%)*	Reference
4/10 (40%)	Hall and O'Toole, 1935
5/8 (63%)	Larson et al., 1978
13/32 (41%)	Cooperstock et al., 1982
16/23 (70%)	Stark et al., 1982
26/29 (90%)	Richardson et al., 1983
21/25 (84%)	Lishman et al., 1984
14/16 (88%)	Mathew et al., 1984
31/50 (62%)	Tabaqchali et al., 1984
46/150 (31%)	Bolton et al., 1984
31/111 (28%)	Karsch et al., 1989
66/90 (73%)	Tullus et al., 1989

* Number of asymptomatic infants and neonates with stool specimens positive for *C. difficile* per number of asymptomatic infants and neonates examined.

changes in the intestinal flora induced by antimicrobial administration (*Onderdonk* et al. 1977; *Mulligan* et al., 1984).

Gnotobiotic Animals

Experiments in gnotobiotic animals further support the importance of the intestinal flora in protecting the host against *C. difficile*-associated intestinal disease (Table 3). When introduced alone into germfree mice, *C. difficile* rapidly establishes a stable population of over 10^8 CFU per ml of caecal contents (*Wilson* et al., 1986; *Onderdonk* et al., 1980). When indigenous mouse, hamster, hare or human intestinal flora is subsequently introduced into the monoassociated mice, *C. difficile* is suppressed to undetectable levels within 3 weeks (*Wilson* et al., 1986; *Raibaud* et al., 1980).

The general success of experiments using complete flora to inhibit the *in vivo* multiplication of *C. difficile* contrasts markedly with the outcome of the use of combinations of bacteria. For example, Wilson and colleagues (*Wilson* et al., 1986) inoculated 150 isolates from the predominant flora of hamsters into gnotobiotic mice pre-colonised with *C. difficile*, causing only a ten-fold reduction in the number of *C. difficile*. This observation suggests that some bacteria important for the suppression of *C. difficile* were still missing from the defined flora or failed to implant in gnotobiotic animals. However, it is also possible that suppression of *C. difficile* is a function of the whole indigenous caecal microflora, a function which cannot be simulated in gnotobiotic studies using a relatively small collection of isolates.

Infant Colonisation

Toxigenic *C. difficile* has been isolated from up to 90% of healthy infants during the first year of life (Table 4). These infants almost invariably remain asymptomatic despite the frequent pres-

ence of high numbers of *C. difficile* and large amounts of toxin in their intestinal tracts. Carrier rates for *C. difficile* fall sharply after the first year of life, although in the second year of life it is still higher than in adults (*Mardh* et al., 1982). Carrier rates for *C. difficile* in healthy adults are reported to be less than 4% (*Rolfe*, 1988; *Bartlett*, 1979).

Asymptomatic intestinal colonisation with *C. difficile* has also been demonstrated in infant hamsters. *Rolfe* and *Iaconis* (1983) challenged hamsters at various ages with 10^7 CFU of a toxigenic strain of *C. difficile*. After 1 day of age, animals were asymptomatically colonised with *C. difficile*, and susceptibility to colonisation continued until the hamsters were 12 to 13 days of age, after which *C. difficile* failed to establish even though the inoculum was large. The development of resistance to *C. difficile* intestinal colonisation correlated with the time at which the hamsters began to sample solid food. The changing diet of the hamster may have resulted in alterations of the intestinal flora, leading to the creation of a restrictive physiologic environment in the intestinal tract.

Neonatal hares are also susceptible to *C. difficile* intestinal colonisation (*Dabard* et al., 1979). Approximately 50% of newborn hares develop a spontaneous and lethal diarrhoeal disease involving *C. difficile* whereas adult hares do not develop this illness. However, neonatal hares inoculated with the adult hare intestinal flora immediately after birth are protected from *C. difficile*-mediated disease (*Dubos* et al., 1984).

The ecological significance of the above studies is that in neonates, *C. difficile* flourishes before the normal intestinal flora has the opportunity to become established. Presumably, the intestinal tracts of infants lack the microorganisms that are normally present in older individuals and that act as a barrier to *C. difficile* colonisation.

INHIBITORY MECHANISMS OF THE NORMAL FLORA

The mechanisms by which the indigenous flora controls *C. difficile* in the intestinal tract are not well understood, although a number of potential control mechanisms have been investigated. There are usually several mechanisms acting in concert to control the population size of a given bacterial species, and it is likely that this is also true for *C. difficile* suppression.

Volatile Fatty Acids

Volatile fatty acids (VFAs) are present throughout the intestinal tract as end products of the fermentation of soluble carbohydrates and other nutrients by members of the intestinal flora. Several investigators have presented experimental evidence that VFAs play an ecological role in the intestinal tract both in modulating indigenous populations and in protecting from colonisation by exogenous pathogens (*Lee* and *Gemmell*, 1972; *Hentges*, 1983). Investigators have examined the role of VFAs in colonisation resistance against *C. difficile*. *Rolfe* et al. (1984) measured the concentrations of VFAs in infant and adult hamsters to determine whether they could account for the observed differences in colonisation resistance against *C. difficile*. The disappearance of *C. difficile* from the caecal contents of hamsters as they aged coincided with the appearance of VFAs at high concentrations. When mixtures of VFAs were prepared in broth at concentrations equal to those present in the caeca of hamsters, there was a direct correlation between the *in vitro* inhibitory activity

of the VFAs and the susceptibility of the hamsters 4 days of age or older to *C. difficile* intestinal colonisation. These investigators postulated that antimicrobial agents may induce *C. difficile* intestinal overgrowth by suppressing the normal intestinal flora components responsible for production of the inhibitory acids. *Hoverstad* et al. (1986) have reported that oral administration of clindamycin to animals leads to faecal VFA concentrations resembling those of germfree animals, indicating severe disturbances in the intestinal microflora. Other investigators, on the other hand, have found no inhibition of *C. difficile* by physiologic concentrations of VFAs (*Borriello* and *Barclay*, 1986). In addition, *Su* and co-workers (1987) presented evidence that VFAs were not involved in colonisation resistance to *C. difficile* in mice.

Competition for Nutrients

Wilson and *Perini* (1988) used a continuous flow culture model of the mouse caecal flora to investigate the possibility that competition for nutrients is one mechanism of colonisation resistance against *C. difficile*. They found that the levels of carbohydrates within a continuous flow culture colonised with mouse intestinal flora were insufficient to support *C. difficile* growth. In particular, it appeared that an unidentified organism competed more efficiently than *C. difficile* for monomeric glucose, N-acetylglucosamine, and sialic (N-acetylneuraminic) acid in the continuous flow culture model.

Suppression of Toxin Production

Some bacterial strains have been shown to prevent mortality due to *C. difficile* not through a strong antagonistic effect of these strains against *C. difficile* but through modulation of cytotoxin production. *Corthier* et al. (1989) reported that mice monoassociated with *C. difficile* died whereas those associated with *C. difficile* and an *Escherichia coli* or *Bifidobacterium bifidum* (both of human origin) survived; the population of *C. difficile* was suppressed a maximum of only ten-fold whereas caecal cytotoxin titres were 1000 times lower than in animals monoassociated with *C. difficile*. The mechanism of modulation of cytotoxin production by these strains has yet to be elucidated.

Competition for Association with Mucosal Surfaces

Mucosal attachment is a prerequisite for successful colonisation of the intestine by both the indigenous microflora and pathogens. *Borriello* and *Barclay* (1985) reported that hamsters previously inoculated with a non-toxigenic avirulent *C. difficile* were protected against lethal effects of a virulent strain of *C. difficile*. It was postulated that the protection afforded, which only occurred if viable cells were administered and allowed to remain in the intestinal tract, was due to competition for attachment sites on intestinal mucosal cells.

Mucin Degradation

Carlstedt-Duke (1990) demonstrated that pre-establishment of a mucin degrading *Peptostreptococcus micros* in germfree mice protected the animals from the lethal effect of subsequent *C. difficile* challenge. It is of interest that the mucin degrading strain of *P. micros* did not prevent diarrhoea resulting from *C. difficile* intestinal colonisation but did prevent death. The mechanisms behind these observations are unknown.

Other Inhibitory Mechanisms

There are several other mechanisms which have been shown to be involved in the control of bacterial populations sizes in the intestinal tract but which have not been examined in *C. difficile*-

associated intestinal disease. These other mechanisms include the lowering of pH and production of hydrogen sulphide, bile acids and/or colicins.

BACTERIOPROPHYLAXIS AND BACTERIOTHERAPY

Because proliferation of *C. difficile* is usually dependent on antibiotic-associated disruption of the intestinal flora that normally would prevent its growth, one approach to treating or preventing *C. difficile*-induced intestinal disease is the addition of microorganisms to the gastrointestinal tract that would restore homeostasis. This is an attractive therapeutic option because it addresses the pathophysiology of *C. difficile*-mediated intestinal disease and avoids the use of antibiotics, which further delays recolonisation by normal colonic flora. To this end various combinations of microorganisms have been used in attempts to inhibit growth of and/or toxin production by *C. difficile in vivo*. Four pro-biotic approaches have been used to treat (i.e., bacteriotherapy) or prevent (i.e., bacterioprophylaxis) *C. difficile*-associated intestinal disease in humans: 1) The use of a complete flora in the form of faecal enemas; 2) The use of combinations of known microorganisms; 3) The use of a non-toxigenic avirulent strain of *C. difficile*; and 4) The use of individual microorganisms. Although all four approaches have been generally successful, the experience is frequently limited and controlled trial data are generally absent.

Faecal Enema

In animal models it is possible to prevent the development of fatal antibiotic-induced ileo-caecitis with daily enemas and orogastric feedings of homogenised caecal contents obtained from healthy animals not receiving antimicrobial agents (*Wilson* et al., 1981; *Larson* and *Welch*, 1993). Rectal infusions of normal faeces have also been effective treatments in several cases of antibiotic-associated PMC in humans (*Schwan* et al., 1984; *Tvede* and *Rask-Madsen*, 1989; *Schwan*, 1989). For example, *Bowden* and colleagues (1978) successfully treated 13 out of 16 patients with PMC using rectal infusions of faeces obtained from normal donors. In total, 21 patients have been treated with faecal enemas and 18 improved. Unfortunately, the role of *C. difficile* in the intestinal disease of the majority of the patients in these studies was unknown. Nonetheless, it appears that faecal enemas may be efficacious for treating disease in humans; however, the degree of risk associated with this approach has not been thoroughly evaluated. There is obviously some concern with giving patients a complex, mixed, undefined flora which could contain a number of potential pathogens.

Bacterial Mixture

Application of faecal material is unpleasant and a preferable therapeutic approach would be to prepare a mixture of the minimum components of the total flora required to confer protection. *Tvede* and *Rask-Madsen* (1989) treated five patients with chronic relapsing *C. difficile* with rectal infusions of a mixture of ten different aerobic and anaerobic bacteria derived from human colonic flora. The mixture led to complete recovery and prompt loss of *C. difficile* and its toxins from the stools of all five patients. Treatment with the bacterial mixture also led to bowel colonisation with *Bacteroides* spp. which had not been present before bacteriotherapy when patients still had symptoms. This observation led the in-

vestigators to speculate that *Bacteroides* may be one of the organisms that provides a natural defence mechanism against intracolonic growth of *C. difficile*.

Non-Toxigenic *Clostridium difficile*

Some strains of *C. difficile* do not produce toxin and when inoculated into clindamycin-treated hamsters do not have the pathogenic potential of the toxigenic strains, although they establish and proliferate in the same manner. Various studies using antibiotic-treated hamsters or gnotobiotic mice have shown that non-toxigenic strains of *C. difficile* have a protective effect against infection by toxigenic strains (*Corthier* and *Muller*, 1988). For example, *Wilson* and *Sheagren* (1983) have shown that prior colonisation of cefoxitin-treated hamsters with a non-toxigenic strain of *C. difficile* increases survival considerably; 26 of 28 hamsters precolonised with a non-cytotoxigenic strain survived subsequent challenge with a cytotoxigenic strain whereas only 6 of 28 survived colonisation with the cytotoxigenic strain alone. The simultaneous administration of both non-toxigenic and toxigenic *C. difficile* did not lead to suppression of toxigenic *C. difficile* and conferred no protection (*Wilson* and *Sheagren*, 1983). In similar independent studies, *Borriello* and *Barclay* (1985) demonstrated that prior colonisation of clindamycin-treated hamsters with non-toxigenic strains of *C. difficile* protected them from subsequent colonisation with a toxigenic pathogenic strain. Protection was not evident if a heat-killed suspension was used or if the colonising non-toxigenic strain was first removed with vancomycin.

Based on the above investigations in animals oral bacteriotherapy with a defined non-toxigenic strain of *C. difficile* would appear to represent an acceptable alternative way to treat hospitalised patients with *C. difficile* diarrhoea. *Seal* et al. (1987) treated successfully two patients with relapsing *C. difficile* diarrhoea following metronidazole and vancomycin therapy with an avirulent strain of *C. difficile*.

Lactobacillus

Studies evaluating gastrointestinal flora and faecal composition during antibiotic therapy have demonstrated a decrease or disappearance of *Lactobacillus* spp. (*Finegold*, 1970; *Finegold* et al., 1967). Based on these observations, a number of investigators have suggested the use of various lactobacillus preparations to reconstitute the normal intestinal flora in patients receiving antibiotics and in patients developing antibiotic-related gastrointestinal problems (*Beck* and *Necheles*, 1961; *Gordon* et al., 1957; *Pearce* and *Hamilton*, 1974).

Lactobacillus GG

Lactobacillus GG is a human lactobacillus strain which has been shown to implant in the intestinal tract and elaborate an antibacterial substance that can inhibit a broad range of bacteria, including *C. difficile* (*Silva* et al., 1987). This strain of lactobacillus has been used to ameliorate successfully relapsing colitis secondary to *C. difficile* diarrhoea in a small number of patients (*Gorbach* et al., 1987).

Lactinex

A commercial preparation of lyophilised *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* (Lactinex; Hynson, Westcott and Dunning, Baltimore, MD) has been shown to be effective in preventing *C. difficile*-induced ileo-caecitis in antibiotic treated hamsters and, significantly, ampicillin associated diarrhoea in humans (*Winans* et al., 1980; *Gotz* et al., 1979). In a

double-blind study, the efficacy of Lactinex in preventing ampicillin-associated diarrhoea in 98 adult patients was studied. Patients were assigned randomly to receive one packet of Lactinex or placebo four times daily for the first five days of ampicillin therapy. The overall incidence of ampicillin diarrhoea in the study was 7.4% which is similar to that observed by others (*Tedesco*, 1975; *Lusk* et al., 1977). All six patients who developed ampicillin-associated diarrhoea were prophylactically treated with placebo. No patients who were prophylactically treated with Lactinex developed diarrhoea secondary to the antibiotic. However, the incidence of *C. difficile* in these patients was not examined.

Saccharomyces boulardii

S. boulardii is a mesophilic, non-pathogenic yeast used in many countries as both a preventive and therapeutic agent for diarrhoea and other gastrointestinal disturbances caused by the administration of antibiotics (*Surawicz* et al., 1989a; *Cano* et al., 1989). This yeast survives transit through the normal human bowel and is unaffected by antibiotic therapy (*Blehaut* et al., 1989; *Boddy* et al., 1991). It can be safely consumed in large numbers and once the agent is discontinued, *S. boulardii* is quickly eliminated from the colon. *S. boulardii* inhibits the growth of a number of microbial pathogens *in vivo* and *in vitro* (*Brugier* and *Patte*, 1975; *Ducluzeau* and *Bensaada*, 1982; *Bizot*, 1955). *S. boulardii* has shown promising results as a probiotic for the treatment and prevention of *C. difficile*-associated disease in experimental animals and in humans.

Animal Studies

Animal studies have indicated that *S. boulardii* protects both hamsters and gnotobiotic mice from *C. difficile* infection (*Toothaker*, 1984; *Massot* et al., 1984; *Castex* et al., 1990). For example, *Corthier* and co-workers (1986) found that a single dose of *S. boulardii* protected 16% of gnotobiotic mice from *C. difficile* infection, whereas 56% were protected when *S. boulardii* was given continuously in the drinking water. *Elmer* and *Corthier* (1991) reported that as the dose of *S. boulardii* was increased from 3×10^8 to 3×10^{10} CFU per ml drinking water, the incidence of survival following *C. difficile* ingestion in germfree mice increased linearly from 0% to 85%. Furthermore, the ability of *S. boulardii* to inhibit *C. difficile* induced intestinal damage was lost if the yeast was given in a non-viable state. Interestingly, no direct antagonistic effect of the yeast on *C. difficile* numbers was detected, whereas a decrease of *C. difficile* toxin production was demonstrated in *S. boulardii* mice.

Recently, *Massot* and colleagues (1984) and *Toothaker* and *Elmer* (1984) have reported that oral administration of *S. boulardii*, initiated before clindamycin exposure, significantly inhibited the growth of *C. difficile* in the caecum and colon and decreased the extent of clindamycin mortality in golden Syrian hamsters. No adverse effects of the yeast treatment were observed in animals receiving *S. boulardii* without clindamycin (*Toothaker*, 1984). Unlike gnotobiotic mice, there was a direct relationship between mortality and the number of *C. difficile* in the caecum and colon. *Elmer* and *McFarland* (1987) demonstrated that *S. boulardii* prevented the development of high counts of *C. difficile* and high toxin titres after cessation of vancomycin treatment in hamsters. The protocol was designed to simulate relapse of human *C. difficile* associated colitis following discontinuation of vancomycin therapy. *S. boulardii* prevented *C. difficile*-induced ileo-caecitis in this model. These inves-

tigators did not determine if the reduction in toxin titres was a result of the lower *C. difficile* counts in yeast treated hamsters or a direct action of *S. boulardii* on the toxins.

Human Trials

Controlled clinical trials in humans have been performed to test the effectiveness of *S. boulardii* as either an adjunctive therapy to antibiotic treatment against *C. difficile* or as the only treatment modality (*Kimmey* et al., 1990; *Surawicz* et al., 1989a; *Surawicz* et al., 1989b). *Surawicz* and co-workers (1989a) examined the effect of *S. boulardii* administration on the incidence of antibiotic-associated diarrhoea in hospitalised patients. *S. boulardii* or placebo was assigned as a concomitant therapy to antibiotics. These investigators found that of the 180 patients examined, 14 of 64 (21.8%) on placebo developed diarrhoea compared with 11 of 116 (9.5%) treated with *S. boulardii*. Of the 48 *C. difficile*-positive patients, five of 16 (31.3%) patients treated with placebo developed diarrhoea compared with three of 32 (9.4%) patients treated with yeast that developed diarrhoea. It is interesting that *S. boulardii* did not appear to prevent *C. difficile* acquisition in these patients.

S. boulardii has also been evaluated for its efficacy in treating recurrences of *C. difficile*-associated colitis in humans (*McFarland* and *Bernasconi*, 1993; *Surawicz* et al., 1989b; *Buggy*, 1985). *Surawicz* and colleagues (1989b) treated 13 patients with recurring *C. difficile* cytotoxin-positive diarrhoea with 10 days of vancomycin and a 30 day course of oral *S. boulardii*. Eleven (85%) patients had no further recurrences. However, only a minority of these patients were positive for stool *C. difficile* toxin and the protective effect was not confined to *C. difficile* culture-positive or toxin-positive individuals. Less dramatic results were obtained in another clinical trial of *S. boulardii* in preventing recurrent episodes of *C. difficile* disease (*McFarland* and *Bernasconi*, 1993). Of the 51 patients with a history of recurrent *C. difficile* disease, 19 of 28 (68%) patients on placebo had another recurrence while 9 of 23 (39%) patients on *S. boulardii* reported a recurrence.

These studies suggest that *S. boulardii* is a safe and effective biotherapeutic agent for the treatment of gastrointestinal disease associated with a specific etiologic agent: *C. difficile*. However, additional prospective controlled clinical trials against *C. difficile*-associated intestinal disease are needed to confirm its efficacy.

Mechanism of Action

Although the exact mechanism of action of *S. boulardii* in protection against *C. difficile*-associated intestinal disease is unknown, the yeast has been shown to both inhibit production of toxins by *C. difficile* and to protect the intestinal mucosa against *C. difficile* toxins (*Corthier* et al., 1986). Studies demonstrate that *S. boulardii* does not have any direct action on the toxins *in vitro* (*Corthier* et al., 1992). However, mice pre-treated with *S. boulardii* survive the administration of a lethal dose of *C. difficile* toxin. The intestinal mucosa of the *S. boulardii* protected mice was not damaged suggesting that the yeast mainly acts on the intestinal mucosa. It has also been shown that the yeast prevents *C. difficile* toxins from damaging intestinal cells in culture (*Czerucka* et al., 1991). *Pothoulakis* and co-workers (1993) demonstrated that pre-treatment of rabbit brush borders with *S. boulardii* reduces toxin A receptor binding in a dose-dependent manner and that pre-treatment of rats with a *S. boulardii* suspension reduces fluid secretion and mannitol permeability

caused by toxin A. The antisecretory effect was mediated by both *S. boulardii* suspensions and filtered supernatants. These investigators concluded that *S. boulardii* was secreting a factor possessing protease activity which enzymatically digests the toxin A receptor on the intestinal mucosa. These investigations suggest that the intestinal cell and not the toxin A molecule itself is an important target for *S. boulardii* protective activity.

Other mechanisms that have been proposed to explain the protective effect of *S. boulardii* include stimulation of the immune system and modification of the toxin A brush border receptor by activation of intestinal enzymes (*Buts* et al., 1986; *Buts* et al., 1990). It has also been suggested that *S. boulardii* might cause a reduction in toxin A receptors as has been reported for the intestinal microflora (*Lucas* et al., 1989).

Other Agents

Streptococcus faecium (*Bellomo* et al., 1980; *Borgia* et al., 1982) and *Bifidobacterium longum* (*Colombel* et al., 1987) have both been effective for the prevention of antibiotic-associated diarrhoea. However, the efficacy of these agents specifically in *C. difficile*-associated diarrhoea is unknown.

CONCLUSIONS

There is a large body of *in vivo* and *in vitro* evidence that components of the normal adult intestinal flora are extremely important in resistance to colonisation by *C. difficile*. Given the complexity of the colonic flora, there is not likely to be one simple explanation for the suppression of *C. difficile*. Nonetheless, since diarrhoea due to toxigenic *C. difficile* primarily occurs because of a loss of normal colonisation resistance in the gastrointestinal tract from antibiotic use, replacing normal flora by bacterioprophylaxis or bacteriotherapy is more logical than prescribing more antibiotics in the prevention and treatment of *C. difficile*-mediated intestinal disease. It is clear that although manipulation of the composition of the colonic flora appears to be a promising approach to the prevention and/or treatment of *C. difficile*-associated intestinal disease, much more work will be required before it can be done on a scientific basis. Furthermore, not all patients who are on antibiotics are susceptible to *C. difficile* infection, and it would be of value to be able to recognise these patients specifically at risk. Because of the low incidence of antibiotic associated diarrhoea (approximately 20%) and the variable intensity of this diarrhoea, it is not practical from a cost/benefit viewpoint to prophylactically treat all patients receiving antibiotic therapy with a probiotic.

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RETHINKING THE ROLE OF PROBIOTICS FOR THE PREVENTION AND TREATMENT OF ENTEROPATHIES

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SUMMARY

In this paper we review the experimental and clinical data indicating that lactic acid producing bacteria of the normal endogenous intestinal microflora strongly affect most functions of immune system, particularly at the level of gut-associated lymphoid tissue (GALT), including the production of cytokines, the mitogen- and antigen-driven proliferation of lymphocytes, the cytotoxicity of natural killer cells, and the production of antibodies. Furthermore, via these mechanisms as well as via exerting a barrier effect, lactic acid producing bacteria counteract the translocation of Gram-negative bacteria from the gut. Furthermore, lactic acid producing bacteria have been shown to mediate the pathogenesis of autoimmunity in experimental models, such as the murine model of Kawasaki disease.

Recent reports have also demonstrated that the administration of lactic acid producing bacteria could prove helpful in the treatment under *in vivo* conditions of enteric infections from pathogens, such as *Salmonella* spp. and rotaviruses. Finally, lactic acid producing bacteria have been shown to reduce mutagenicity in some experimental models, therefore suggesting a role for probiotics in the prevention of malignancy.

INTRODUCTION

The endogenous bacterial microflora have a key role with regard to many metabolic functions and in natural resistance to infections from several pathogens, mostly in the gastrointestinal tract.

A body of evidenc indicates that lactic acid producing bacteria, which mainly account for the endogenous intestinal microflora, may strongly affect most functions of the immune system, particularly at the level of the gut-associated lymphoid tissue (GALT), including the production of cytokines, the proliferation of lymphocytes following mitogens and antigens, the cytotoxicity of natural killer (NK) cells, the production of antibodies, and the metabolic and phagocytic functions of macrophages.

Here we will briefly review the physiology of endogenous intestinal microflora and the most common and significant conditions leading to distur-

bances in the normal intestinal microecology. Furthermore, since a strict relationship linking foods containing lactic acid producing bacteria, such as yoghurt, the endogenous bacterial microflora, and the regulation of immune response toward pathogens has been suggested, we will summarise both clinical and experimental reports emphasising the use of lactic acid producing bacteria in therapy and immunomodulation of human diseases. Overall, the reviewed data indicate that lactic acid producing bacteria could be a promising tool for ecological therapy of mucosal diseases as well as for the development of original and flexible vectors for targeting in the gastrointestinal tract.

CAUSES OF MICROFLORA DISTURBANCES

The most common and significant cause of disturbances in the normal endogenous intestinal microflora is the administration of antimicrobial agents (*Lidbeck* and *Nord*, 1993; *Nord*, 1993). The administration of these agents may seriously disturb the balance of the normal intestinal microflora. This disturbance can cause bacterial overgrowth and the emergence of resistant microorganisms which may lead to serious infections and also encourage the transfer of resistance factors among bacteria.

Many antimicrobial agents, including penicillins, cephalosporins, monobactams, carbapenems, macrolides, clindamicin, tetracyclines, nitroimidazoles and quinolones, may have ecological effects on the human intestinal microflora (*Lidbeck* and *Nord*, 1993; *Nord*, 1993).

Laboratory animals are a valuable model which allows us to evaluate the effects of antimicrobial administration upon intestinal bacterial microflora. For example, in mice the selective elimination of aerobic Gram-negative bacteria by oral polymixin, aztreonam or temocillin resulted in a reduction of the endotoxin concentration of faecal supernatants to 10% of untreated controls (*Goris* et al., 1986). In addition, further decrease of the endotoxin concentration to 1% was achieved by total decontamination of the intestinal tract by oral cephalotin/neomycin treatment (*Goris* et al., 1986).

In an experimental rat model (*Minelli* and *Benini*, 1993), fluoroquinolones proved able to reduce the levels of enterobacteria, while Gram-positive bacteria (enterococci, staphylococci, lactobacilli) were little affected. It is worth noting that comparable effects were observed after intraperitoneal and oral administration of oral pefloxacin. The changes induced by fluoroquinolones on intestinal flora showed a uniform trend: certain differences may be ascribed to different pharmacokinetic properties such as bioavailability and metabolism. In this model, parenteral imipenem caused a significant decrease in the mean concentrations of *E. coli*, clostridia, and fungi, whereas aztreonam induced a marked and prompt inhibition of *E. coli* and *Proteus* spp. The prolonged treatment, in turn, induced an overgrowth of fungi and bacteroides. Teicoplanin caused a significant decrease in the clostridia and anaerobic lactobacilli. These results are closely comparable to those observed in humans (*Minelli* et al., 1993).

The potential of an antimicrobial agent to change the intestinal microflora is related to its antibacterial activity, route of administration and pharmacokinetic properties, such as incomplete absorption of any orally administered antibacterial compound, secretion in the

bile or from the intestinal mucosa (*Lidbeck* and *Nord*, 1993; *Nord*, 1993). Notably, in most cases the parenteral route induced changes in the intestinal ecosystem as did oral administration.

In subjects with gastrointestinal inflammation (gastritis, duodenitis, enteritis, cholangitis, cholecystitis) the microbial flora may be qualitatively and/or quantitatively modified with no clinical and functional repercussions (*De Simone* et al., 1993). However, after episodes of acute diarrhoea frequently one could find a reduction of anaerobic agents concurrent with the causative agent (*De Simone* et al., 1993).

Also in patients with inflammatory bowel disease, most notably ulcerative colitis, the faecal microflora undergoes changes (*Yamamura*, 1987). In fact, the counts of obligate anaerobes were significantly decreased irrespective of stool condition, stage, and severity of the disease. The frequencies of detection of obligate anaerobes were much lower particularly in severe cases and cases with bloody diarrhoea. Specifically, the counts and frequencies of detection of bifidobacteria reflected the severity of the disease (*Yamamura*, 1987). Furthermore, in the genera of the Enterobacteriaceae *E. coli* was not isolated and *Proteus*, which was not isolated from the faeces of healthy individuals, was often isolated and became dominant in severe cases (*Yamamura*, 1987). These findings clearly point out the presence of abnormal microflora in the gut of patients with ulcerative colitis. In addition, facultative anaerobes, such as streptococci, were increased more markedly in first relapsing cases as compared with untreated cases (*Yamamura*, 1987).

High doses of radiotherapy may cause an abnormal proliferation of bacteria, chiefly focal, which is related to the absorbed amount of radiation (*De Simone* et al., 1993).

In liver cirrhosis, it has been demonstrated that an abnormal bacterial increase in the stool is associated with poor absorption (*De Simone* et al., 1993). In these patients, the microflora of the intestine acquires pathogenic properties because of the products of its metabolic degradation. In fact, due to the reduced liver capability of detoxification the amino acid metabolites (ammonia, indoles, phenols, amines) pass through systemic circulation without being detoxified properly. It remains to be established whether the bacteria in the intestine of patients with liver cirrhosis are able to produce larger amounts of ammonia than in other conditions of increased bacterial proliferation or, in turn, this phenomenon could be related to a percent increase in *Klebsiella* and *Proteus* counts which contribute to a great extent to the derangement in urea metabolism.

Finally, since enteric infections have been implicated in the heterogeneous pathogenesis of the irritable bowel syndrome (IBS), which includes factors ranging from psychoneurotic behaviour and emotional stress to dietary fibre deficiency and food intolerance, and there is evidence of an increase in the inflammatory cells present in the gut of some IBS patients (*Collins*, 1992; *Whitehead* et al., 1992), it could be hypothesised that the immune reactivity toward modification in the endogenous intestinal microflora could have a role in the pathogenesis and pathophysiology of at least a subpopulation of IBS patients.

COLONISATION RESISTANCE AND LACTIC ACID PRODUCING BACTERIA.

Low *Bacteroides* counts in the stool, for instance, have been correlated with an increased susceptibility to infection from *Salmonella enteritidis* as well as combined *Escherichia coli*, whereas *Proteus mirabilis* and *Streptococcus faecalis* have been shown to antagonise *Vibrio cholerae* (*De Simone* et al., 1993). The protection afforded by microflora against intestinal colonisation with pathogens is referred to as colonisation resistance. It is worth noting that this mechanism of natural resistance is involved also in protecting the host against fungal infections, in addition to bacteria, as shown by the resistance afforded by *E. coli* toward *Candida albicans*, regardless the immunocompetence of the host (*De Simone* et al., 1993).

The healthy human body harbours an extensive number of microrganisms that inhabit surfaces and cavities exposed or connected to the external environment. It is estimated that the intestinal microflora of any given individual contains more than 400 species of bacteria. The major bacterial populations are located in the large intestine where the bacterial concentration is 10^{11} to 10^{12} CFU/ml of faecal material (*Simon* and *Gorbach*, 1986). Notably, the microflora is not a static population but its composition is the result of host physiology, microbial interactions, and environmental influences, including the steroid sex hormone pattern and diet (*Finegold* et al., 1974; *Marsh* et al., 1992; *Minelli* et al., 1993).

Although the host plays a direct role in colonisation resistance by desquamation of mucosal cells, secretion of saliva or mucus, and finally by swallowing or peristalsis, a relevant contribution of the host may come more indirectly both through the gut-associated lymphoid tissue (GALT) and endogenous bacterial microflora. Recently, it has been tentatively concluded that the GALT may not only respond positively to foreign (bacterial) antigens by producing specific IgA but also by forming specific suppressor cells. The induction of specific unresponsiveness (tolerance) to certain microrganisms may explain the stability of the composition of the endogenous flora. It may also explain the difference in intestinal endogenous flora between individuals of the same species. This hypothesis is supported, at least partly, by the finding that during circumstances of significantly reduced thymus function since birth, important components of the endogenous flora and thus of colonisation resistance are apparently inhibited (*van der Waaij*, 1985). This, and the malfunction of the T cell-depleted GALT, allows potentially pathogenic microbes to colonise the gut in abnormally high numbers and great diversity, a condition which is associated with a clinical syndrome called "wasting disease".

Furthermore, recently considerable evidence has been accumulated showing that various members of physiological microflora liberate low molecular weight peptides which, apparently, are essential for adequate immune responses of the host (*Pulverer* et al., 1993). The antibiotic decontamination (e.g. of the BALB/c mouse intestinal tract) results in a lack of generation of immunopriming microbial peptides leading to immunosuppression (*Pulverer* et al., 1993). The biochemical analysis revealed reproducible chromatographic fractions which selectively influence maturation, proliferation, and activation of immune cells (*Pulverer* et al., 1993).

BACTERIAL TRANSLOCATION AND LACTIC ACID PRODUCING BACTERIA.

Bacteria constituting the endogenous intestinal microflora may pass still alive through the gastrointestinal lumen and reach the local lymphatic organs (Peyer's patches, mesenteric lymph nodes) as well as other organs (liver, spleen, blood). This phenomenon occurs more frequently with Gram-negative bacteria and facultative anaerobes (*E. coli*, *P. mirabilis*, *K. pneumoniae*) than with obligate anaerobes and Gram-positive bacteria. Such a phenomenon does not occur under physiological conditions when an intact endogenous intestinal microflora is present and the host is fully immunocompetent (*De Simone* et al., 1993). Usually, viable endogenous bacteria are not detectable in the organs of healthy gnotobiotic animals since the translocating microrganisms are killed while in transit or upon arrival in the reticuloendothelial organs (*Berg*, 1988). Since endogenous bacteria translocate from the gastrointestinal tract at higher rates in neonatally thymectomised mice or nu/nu mice than in nu/+ or thymus-grafted nu/nu mice it was postulated that a high caecal population level of a given strain is not the unique prerequisite for translocation (*Steffen* et al., 1988). In addition, it was shown that immunosuppressive and antimicrobial agents, thymectomy, endotoxin, tumours, diabetes, protein malnutrition, and thermal injury can promote translocation (*Walken* and *Owen*, 1990). Furthermore, in experimental animal models bacterial translocation was found following small bowel transplantation and in animals with graft-versus-host disease (GvHD), with *Staph. epidermidis* being the most prevalent organism (Price et al., 1993). There was a large increase of *Staph. epidermidis* mostly in animals with transplant rejection. These findings demonstrate that GvHD and transplant rejection are associated with shifts in intestinal microflora toward potentially pathogenic organisms and that bacterial translocation leads to a major threat for the development of sepsis.

As a rule, bacterial translocation is due to an altered permeability of the intestinal epithelium after stress, unapparent infections, tumours or to the host's immunosuppression (*Walken* and *Owen*, 1990). Therefore, the bacterial translocation may be considered as either a deficiency of the mucosal barrier in confining the bacteria to the gastrointestinal lumen or a deficiency of the immune system and non-specific defences in clearing and killing bacteria which cross the intestinal barrier or both. This phenomenon could have a key role in the pathogenesis of opportunistic infections due to endogenous intestinal bacteria, mostly in debilitated patients and/or those with tumours, during long-term treatment with either immunosuppressive drugs or antibacterial compounds or both.

It is important to know whether certain foods or bacterial species are involved in modulating bacterial translocation from the gut. For example, commercially available chemically defined liquid diets result in altered intestinal microflora and bacterial translocation from the gut (*Alverdy* et al., 1990) whereas enteral feeding with peptide nutrients has been shown to improve the mucosal barrier against microrganisms (*Alexander*, 1990).

Recent experiments from our groups have shown that *Lactobacillus bulgaricus*, one of the lactic acid producing bacteria present in yoghurt, which has the capability to adhere to the intestinal mucosa (*Bianchi Salvadori* et al., 1984; *Bianchi Salvadori*, 1986), is pivotal in

inhibiting translocation of Gram-negative bacteria present in the gut (*De Simone* et al., 1992). According to our results, the barrier effect exerted by *L. bulgaricus* against *E. coli* translocation approximated >70%. However, our data rule out a simple barrier effect played by *L. bulgaricus* and suggest that *L. bulgaricus* boosts the host's immune defences against translocated *E. coli* (*De Simone* et al., 1992).

MODULATION OF CYTOKINE PRODUCTION BY LACTIC ACID PRODUCING BACTERIA

Endogenous bacterial microflora, mostly lactic acid producing bacteria, have been shown to strongly modulate the cytokine network which regulates the immune response and drives effector arms toward invading pathogens. For example, peritoneal macrophages from normal mice produced significantly more IL-1 and IL-6 *in vitro* than those of germfree mice (*Nicaise* et al., 1993). Furthermore, IL-1 and IL-6 production from germfree mice implanted with *E. coli* was as comparable as in normal mice (*Nicaise* et al., 1993). In turn, *Bifidobacterium bifidum* did not increase the production of these two cytokines (*Nicaise* et al., 1993). In these experiments, TNF-α was produced only by peritoneal macrophages from normal mice and germfree mice implanted with *E. coli* (*Nicaise* et al., 1993).

These data, overall, suggest that Gram-negative bacteria are the most efficient stimulus for driving the production of macrophage derived cytokines. Also, it is worth to note that bacterial flora stimulated cytokine production soon after implantation. In addition, recent reports have demonstrated that *L. acidophilus* induces the production of IFN-α/β by murine peritoneal macrophages (*Kitazawa* et al., 1993), therefore suggesting that the inducing activity of IFNs may be one of the available biologic parameters for designating the diary products containing *L. acidophilus* as physiologically functional foods. Both of the bacteria commonly found in yoghurt (*L. bulgaricus* and *Streptococcus thermophilus*) have been shown to induce the production of IL-1 β, TNF-α, and IFN-γ, but not of IFN-α and IL-2, by peripheral blood mononuclear cells (PBMCs) from humans (*Pereyra* et al., 1993). Furthermore, the walls from these bacteria, but not their cytoplasm, induced a comparable cytokine production (*Pereyra* et al., 1993). These cytokines were also induced by *L. casei*, *L. acidophilus*, *Bifidobacterium* spp., and, to a lesser extent, *L. helveticus* (*Pereyra* et al., 1993). Notably, in this report the IFN production was estimated by the 2-5 synthetase activity from PBMCs following a single ingestion of bacteria in yoghurt or sterile milk and the activity of the yoghurt group enzyme was about 80% higher than that of the milk group; however, no cytokine was detectable in the serum (*Pereyra* et al., 1993).

In a recent report from our laboratory, we have shown that the addition of small quantities of yoghurt containing live *L. bulgaricus* and *S. thermophilus* to ConA-driven human PBMCs resulted in a strong enhancement of IFN-γ production (*De Simone* et al., 1993). Furthermore, these supernatants augmented NK cell cytotoxicity against K562 targets much more with respect to control supernatants from PBMC cultures stimulated with ConA only (*De Simone* et al., 1993). Similar results were found by using *L. aci-*

dophilus, *L. casei*, and *L. plantarum* (*De Simone* et al., 1986). Our hypothesis was that lactic acid producing bacteria induce PBMCs to produce cytokines, such as IL-1 and IL2, which activate resting NK cells to synthesise and release IFN-γ, proliferate, and exert cytotoxicity. This hypothesis has been further supported by experiments demonstrating the binding of lactic acid producing bacteria to both CD4 and CD8 cells (*De Simone* et al., 1993; *De Simone* et al., 1988a). As seen in the case of *Salmonella*-stimulated lymphocytes (*De Simone* et al., 1986; *Antonaci* and *Jirillo*, 1985; *De Simone* et al., 1988a), the binding of lactic acid producing bacteria to T lymphocytes should be referred to as a potent stimulus for immune cell activation.

Recently, our group has shown that also the administration of lactic acid producing bacteria under *in vivo* conditions may strongly enhance the production of IFN-γ. In our study, healthy volunteers have received lyophilised dietary lactobacilli (3×10^{12} microorganisms) and 200 g of plain yoghurt at 24 hours intervals for 28 days. The control group received skimmed milk in a quantity calorically equivalent to that of the yoghurt group. Our results were that by feeding large quantities of dietary lactic acid producing bacteria, a strong increase in the serum levels of IFN-γ as well as the expansion of both B lymphocytes and NK cells can be attained in the normal host (*De Simone* et al., 1993).

However, we have data obtained in an experimental model of *Cryptosporidium parvum* infection indicating that also mechanisms other than the increased production of IFN-γ are involved in mediating the increased resistance toward pathogens induced by lactic acid producing bacteria.

C. parvum is a protozoan parasite that causes diarrhoeal disease in a variety of mammals, including humans and economically important livestock species (*Fayer* et al., 1990). The disease is especially severe in immunocompromised hosts and has become a major cause of morbidity and mortality among patients with the acquired immunodeficiency syndrome (AIDS) (*Ungar*, 1990). Mechanisms of immunity to *C. parvum* are not well understood (*Zu* et al., 1992), but several *in vivo* studies suggest that both CD4 lymphocytes and IFN-γ are critical in resistance and recovery from C. parvum infection (*Ungar* et al., 1991; *Chen* et al., 1993; *Chen* et al., 1993), in addition to endogenous bacterial intestinal microflora. In fact, it is worth to note that germfree adult mice are more susceptible to the primary challenge than normal mice and while severe combined immunodeficient (SCID) mice are relatively resistant to *C. parvum* infection, germfree SCID mice are highly susceptible (*Harp* et al., 1992). Therefore, the presence of intestinal microflora strongly influences mice susceptibility to *C. parvum* infection.

The results of our experiments support the hypothesis that the colonisation of the gut of germfree mice with lactic acid producing bacteria can protect them from *C. parvum* infection (*Harp* et al., submitted). In fact, we found that germfree mice colonised with lactic acid producing bacteria were clearly less infected with *C. parvum* than controls without lactic acid producing bacteria (*Harp* et al., submitted). However, this protection was not directly correlated with induction of IFN-γ by lactic acid producing bacteria (*Harp* et al., submitted) since the two groups challenged with *C. parvum* both produced mRNA for IFN-γ despite the fact that mice colonised with lactic acid producing bacteria were protected from infection and the non-colonised group was not (*Harp* et al., submitted). In addition, in

our experiments germfree mice treated with lactic acid producing bacteria only did not produce message for IFN-γ (*Harp* et al., submitted). Therefore, the colonisation of germfree mice with lactic acid producing must be protecting either via some mechanisms not involving IFN-γ or through an indirect pathway. Additionally, it is possible that IFN-γ message and/or protein was induced earlier in animals receiving lactic acid producing bacteria only and disappeared by time of necropsy while the message persisted in mice injected with both lactic acid producing bacteria and *C. parvum*. It is further possible that the group injected with *C. parvum* only was not protected from challenge because the induction of IFN-γ seen in this group was a late event occurring as a result of infection and was not sufficient to prevent colonisation with the parasite.

The implications of our findings in SCID mice with experimental *C. parvum* infection in other species, such as humans with AIDS, are not still clear. It is interesting that the apparent age-related susceptibility and development of resistance in calves and immunocompetent mice correlates with the acquisition of intestinal microflora (*Harp* et al., 1990). Similarly, one may speculate that the immunocompromised state of AIDS patients, often coupled with poor nutritional status and extensive antibiotic therapy, may result in an altered intestinal microflora which then contributes to the increased susceptibility to *C. parvum* infection. In addition preliminary data from our laboratory suggest that the treatment of *C. parvum* infected AIDS patients with lactic acid producing bacteria may be of some benefit in alleviating symptoms (manuscript in preparation).

MODULATION OF GUT-ASSOCIATED LYMPHOID TISSUE BY LACTIC ACID PRODUCING BACTERIA.

A body of evidence indicates that physiological endogenous microflora has a key role to allow an appropriate development of mucosal immune system (*De Simone* et al., 1993). It has been suggested that this effect of microflora may be mediated by the adhesion of bacteria to gut-surface epithelium, which results in the active stimulation of GALT.

In this regard, we have shown that lactic acid producing bacteria are able to modulate several functions of GALT. In fact, we found that PP cell suspension cultures from BALB/c mice fed yoghurt containing living lactic acid producing bacteria exhibited a strong increase of blastogenic proliferative responses to mitogens such as phytohaemagglutinin (PHA) and LPS compared to controls (*De Simone* et al., 1993). Furthermore, the increased cell proliferation to LPS, which is mainly a mitogen for B lymphocytes, was correlated with an expansion of B lymphocyte pool in PP (*De Simone* et al., 1993). These latter data have been further supported by a recent report demonstrating that the content of immunoglobulin-synthesising cells in the jejunal lamina propria of germfree mice was significantly increased following oral and intraperitoneal administration of killed *L. acidophilus* strains (*Smeyanov* et al., 1992).

We observed results as comparable as in PP when the mitogen driven splenocyte proliferation was assayed. In fact, mice fed living lactic acid producing bacteria had a strong increase of splenocyte proliferation to lectins, including both mitogens for T cells (PHA,

ConA) and B cells (PWM) (*De Simone* et al., 1993). These effects were correlated with an expansion of T lymphocyte pool in the spleen (*De Simone* et al., 1993). In addition, we found that feeding lactic acid producing bacteria resulted in increased serum levels of IgM and IgG2a (*De Simone* et al., 1993). Furthermore, a brief treatment with heat proved sufficient to strongly reduce these effects of yoghurt, suggesting that its immunomodulating properties are strictly dependent on the presence of viable lactic acid producing bacteria (*De Simone* et al., 1993).

MODULATION OF MACROPHAGE FUNCTIONS BY LACTIC ACID PRODUCING BACTERIA

It is worth noting our finding in mice fed living lactic acid producing bacteria of increased production of oxygen metabolites by splenocytes following zymosan stimulation (*De Simone* et al., 1993; *De Simone* et al., 1988b) as well as the report of *Smeyanov* and coworkers (1992) showing that the oral and intraperitoneal administration of *L. acidophilus* to germfree mice lead to a significant rise in the level of luminol-dependent chemiluminescence of peritoneal macrophages. These data are in agreement with the hypothesis that lactic acid producing bacteria enhance both the phagocytic activity and the respiratory burst of monocyte-macrophage cells, probably resulting to enhance both phagocytosis and killing of pathogens. Moreover, the possibility that lactic acid producing bacteria upmodulate the functions of monocytes-macrophages as antigen-presenting cells should be considered. Further studies are required to better understand this issue.

MODULATION OF RESISTANCE TOWARD *SALMONELLA TYPHIMURIUM* BY LACTIC ACID PRODUCING BACTERIA

The demonstration that lactic acid producing bacteria are able to inhibit under *in vitro* conditions the growth of food-borne pathogens, including *Salmonella typhimurium*, (*Gilliland* and *Speck*, 1977) prompted to evaluate the mechanisms accounting for this antibacterial effect.

Natural antibiotics synthesised by lactic acid producing bacteria have been identified and, notably, bulgarican which is produced by *L. bulgaricus*, has been shown to possess a wide spectrum of *in vitro* antibacterial activity (*Reddy* et al., 1984). In addition, live microbial therapy has been shown in some reports to be more effective than the administration of antibiotics for treating infections from *Salmonella* spp. (*Hitchins* et al., 1985). However, these studies did not assay whether the protective effects of lactic acid producing against *Salmonella* infections are due to the enhancement of specific immune response.

Protection toward *Salmonella* is mediated in the early phase of infection by macrophages and by specific immunity in the late phase of infection (*Dichelte* et al., 1984; *Akeda* et al., 1981).

We have recently shown that yoghurt containing viable lactic acid producing bacteria strongly enhances murine defences against *S. typhimurium* via several mechanisms (*De Simone* et al., 1988c; *De Simone* et al., 1993), as

shown by:

a) increased antibacterial activity against *S. typhimurium* of PP mononuclear cells. Furthermore, this effect seems to be mediated, at least in part, by IgA antibodies. This increased activity is pivotal for inducing the host resistance toward invading salmonellae since their virulence is related to the ability to survive and multiply within PP microenvironment (*Dichelte* et al., 1984);
b) strongly increased absolute numbers of phagocytising macrophages, probably due to the accumulation of migratory macrophages from the pool of circulating monocytes at the sites of infection;
c) the strongly increased proliferative responses of splenocytes to both T cell and B cell mitogens, such as ConA and LPS.

Overall, these immunomodulating effects of administering viable lactic acid producing bacteria resulted in a strong reduction of *S. typhimurium* growth in both spleen and liver, therefore accounting for the higher survival rate of animals treated with viable lactic acid producing bacteria.

MODULATION OF AUTOIMMUNITY BY LACTIC ACID PRODUCING BACTERIA

The induction of coronary arteritis in mice by *L. casei* cell wall is thought to represent an animal model of the Kawasaki disease (*Tomita* et al., 1993). In fact, under *in vitro* conditions the treatment of vascular endothelial cells with supernatants from human PBMCs stimulated with *L. casei* cell wall has been shown to both enhance the adherence of polymorphonuclear cell (PMNs) to human endothelial cells and increase the expression of intercellular adhesion molecule-1 (ICAM-1) (*Tomita* et al., 1993). Notably, supernatants contained high concentrations of TNF-α and PMN adherence correlated directly with the concentration of TNF-α and both ICAM-1 expression and enhanced PMN adherence were inhibited by anti-TNF-α treatment (*Tomita* et al., 1993). In addition, the initial coronary inflammatory reaction in the mouse model has been shown to involve PMN adherence to vascular endothelium that has been activated by TNF-α released by PBMCs following stimulation with *L. casei* cell wall (*Tomita* et al., 1993).

LACTIC ACID PRODUCING BACTERIA IN THE TREATMENT OF DISEASES

Lactic acid producing bacteria are part of the normal Gram-positive anaerobic microflora. Through the production of lactic and acetic acids, hydrogen peroxide, and antimicrobial substances, such as bacteriocin-like compounds, these microorganisms contribute substantially to the maintenance of colonisation resistance, mostly against *Listeria monocytogenes*, *E. coli*, *S. typhimurium*, and *S. enteritidis* (*Fernandes* et al., 1988; *Lidbeck* and *Nord*, 1993; *Chateau* et al., 1993)). Therefore, it is considered important to maintain or increase the levels of lactic acid producing bacteria in the intestinal microflora to favourably alter the microecology of the gut and inhibit the growth of pathogenic bacteria. In fact, a body of evidence from both experimental and clinical

studies indicates that the administration of lactic acid producing bacteria could lead to significant changes in the intestinal microflora population (*Fernandes* et al., 1988; *Johansson* et al., 1993; *Kafarskaya* et al., 1993). However, some reports suggest that lactic acid producing bacteria should be taken continuously in order to maintain high levels of these protective bacteria in the small intestine (*Lidbeck* et al., 1987).

Furthermore, it is well known that disturbances in the normal intestinal microflora leads to gastrointestinal disorders often resulting in diarrhoea (*Fernandes* et al., 1988; *Johansson* et al., 1993). In addition, the colonisation of the gastrointestinal tract by foodborne pathogenic bacteria is as a rule correlated with a strong decrease in the counts of endogenous intestinal lactic acid producing bacteria (*Fernandes* et al., 1988; *Johansson* et al., 1993)

The ability of lactic acid producing bacteria to affect both systemic and mucosa-associated immune response, as reported above, suggests that the administration of lactic acid producing bacteria could strongly contribute to promote the recovery from infections. For example, *Lactobacillus* spp. strain GG has been recently shown to promote the recovery of children with rotavirus diarrhoea via augmenting both local and systemic immune defence (*Kaila* et al., 1992). Furthermore, specific IgA response has been endorsed, which is probably relevant in allowing protection against rotavirus reinfections (*Kaila* et al., 1992). However, a systemic immune response to lactic acid producing bacteria has been demonstrated to be triggered by their interaction with intestinal mucosa (*Takahashi* et al., 1993). Whether this phenomenon could be detrimental for the therapeutic efficacy of administering lactic acid producing bacteria to patients with intestinal infections, such as rotavirus diarrhoea, remains to be established.

Currently available literature suggests that, in addition to the prophylaxis of intestinal and urogenital infections, potential beneficial effects of lactic acid bacteria include the prophylaxis and the treatment of lactose maldigestion, cholesterol metabolism, and diarrhoeal disorders (*Marteau* et al., 1993).

Finally, lactic acid producing bacteria could have a role in the surveillance against tumours, as suggested by recent experiments assaying urinary mutagenicity in humans (*Hayatsu* et al., 1993). The effect of 3-week oral administration of *Lactobacillus casei*, which is commonly found in yoghurt, on the urinary mutagenicity derived from the ingestion of fried ground beef has been evaluated. The comparison of the urinary mutagenicity found before and after the *L. casei* treatment showed that the treatment resulted in a strong decrease of mutagenicity (*Hayatsu* et al., 1993). This suppressing effect is likely to be related to the changes in the intestinal microflora population induced by *L. casei* supplementation. Further studies are required to evaluate the possible role of administering lactic acid producing bacteria in the prevention of malignancy.

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INVOLVEMENT OF CD LYMPHOCYTES IN THE PRODUCTION OF INTERFERON INDUCED BY NON-PATHOGENIC BACTERIA

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SUMMARY

The effects of non-pathogenic bacteria on the production of different cytokines by circulating mononuclear cells from healthy subjects were studied *in vivo* and *in vitro*. Feeding a diet containing fermented dairy products, induced a significant increase of 2-5A synthetase activity in these cells suggesting a production of interferon. *In vitro*, mononuclear cells produced interferon-γ in presence of lactic bacteria like *Lactobacillus acidophilus*, *Streptococcus lactis* and *Streptococcus thermophilus*. *Streptococcus thermophilus* induced also the production of interleukin-1β. All the studied bacteria, including *Bifidobacterium* and *Lactobacillus casei* induced the production of interleukin 6. When incubated in presence of a monocyte depleted lymphocyte population, the production of interferon-γ was reduced by 76% when an anti-CD4 co-receptor was added in the medium, and by 62% in presence of an anti-CD8 co-receptor. The data indicates that non-pathogenic bacteria were susceptible to induce the production of different cytokines depending on the species and that the mechanism involved the two pathways of major histocompatibility complex.

INTRODUCTION

The possibility for non-pathogenic bacteria, to induce or stimulate immune functions, has been poorly studied. It has been suggested however that the gut flora may play a role as shown by higher levels of 2-5A synthetase, a marker of the production of interferon (IFN), in circulating mononuclear cells of conventional mice compared to germfree mice (*Galabru* et al., 1985). Indeed, it has been shown *in vitro*, that non-pathogenic bacteria from the human gut flora are able to induce the production of different cytokines (*Solis Pereyra* and *Lemonnier*, 1993; *Rusch*, 1994). In healthy subjects, both *in vivo* and *in vitro*, we have shown an increased production of IFN induced by bacteria from yoghurt, this effect being due to their walls (*Solis Pereyra* and *Lemonnier*, 1991, 1993). Interestingly, feeding 10^{11} lactic bacteria from yoghurt increased the 2-5A synthetase activity of circulating mononuclear cells from healthy subjects (*Solis Pereyra* and *Lemonnier*, 1991). This would indicate that consuming non-pathogenic bacteria can stimulate the production of IFN and that this production is added to that spontaneously observed in healthy subjects.

In this work, we have compared dif-

ferent species of dairy bacteria on their ability to induce the production of different cytokines by human mononuclear cells. The production of IFN-γ was studied in healthy subjects after a regular ingestion of yoghurt and the involvement of class I and class II histocompatibility systems was investigated.

METHODS

In vivo study

Samples of blood mononuclear cells (BMC), for 2-5A synthetase activity determination, were prepared from blood of 8 healthy subjects who were included in a cross-over protocol during two periods of 15 days each: during which, they were asked either to consume at least one yoghurt per day or no yoghurt. Samples of blood were taken at the end of each period.

2-5 A synthetase activity in BMC

Cell extracts were prepared by resuspending the BMC in 100 µl lysis buffer: 10 mM Hepes buffer pH 7.6, 10 mM KCl, 2 mM Mg (OAc)2, 4 H_2O, 7 mM 2-mercaptoethanol, with 0.5% Nonidet P-40, incubating the suspension at 4°C for 10 min and centrifuging 10 min at 5000 g. The cytosol was used immediately for the enzyme assay.

Cytoplasmic activity of 2-5A synthetase was determined as described previously (*Hovanessian* et al., 1977, *Justensen* et al., 1980). Briefly poly(rI):(rC)-agarose beads (Pharmacia Fine Chemicals, Uppsala, Sweden) were washed with several volumes of 20 mM Tris-HCl pH 8,5 mM Mg(OAc)2, 1 mM dithiothreitol, 25 mM KCl, 10% glycerol, 1 mM EDTA, (buffer D), and 30 µl aliquots were placed in microtiter tubes. Aliquots (100 µl) of cytosol, were mixed with the beads and incubated for 15 min at room temperature. The beads were washed three times with buffer D and all liquid carefully removed. The beads were then incubated with 10 µl reaction mixture: 25 mM Mg(OAc)2, 0.25 mg/ml bovine serum albumin, 12 µg poly(rI):(rC), 7 mM ^{32}ATP (50 mCi/ml > 15 TBq/mmol, Amersham, UK), 0.25 mg/ml creatin kinase, 0.01M creatin phosphate, in 20 mM Tris-HCl buffer pH 8 for 2 hours at 37°C. The reaction was stopped with 20 µl of 50 mM EDTA and 6 µl samples of incubation mixture were spotted onto PEI cellulose plate. The plates were chromatographed for 16 hours in 2 M Tris-HCl, pH 8.6 to separate 2-5 A oligoadenylates from ATP. The radioactive spots were located using X Ray film, cut out, collected in scintillation liquid and counted in the ^{32}P-channel of a scintillation counter. The percentage conversion of ATP to oligoadenylates was calculated as pmoles ATP incorporation/min per 10^7 cells. Tubes containing cell extracts from were BMC stimulated with IFN and tubes containing no cell extract were included in each assay to minimise the variations due to the batch of ^{32}P ATP used or other experimental parameters.

Preparation of cells and cultures

Human blood was drawn in heparin (30 U/ml) from healthy donors, the mononuclear cell fraction (BMC) was obtained by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, NJ) (*Boyum*, 1968). BMC were washed 3 times with phosphate buffered saline and counted. In the second part of this work, IFN-γ was the sole cytokine studied. This cytokine being produced by T lymphocytes, monocytes depleted lymphocytes population (LDM) were obtained by incuba-

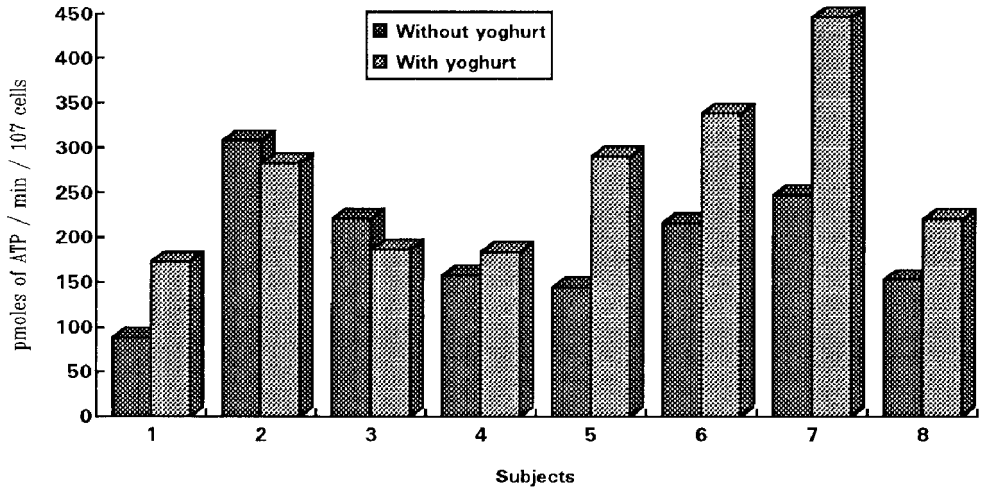

Figure 1: 2-5 synthetase activity of blood mononuclear cells from healthy subjects consuming a diet containing or not at least a yoghurt per day during 2 periods of 15 days (n=8)

tion of BMC with L-leucine methyl ester (Leu-OMe, Sigma, France): Leu-OMe was dissolved in phosphate buffered saline (PBS, Flow Laboratories, Irvine, UK), and filtered through a 0.45 µm filter immediately before use. Cells were suspended in PBS at concentration of $5x10^6$ cells/ml. They were incubated in 17x100 mm polypropylene culture tubes (Becton Dickinson, Rutherford, NJ) with 5 mM Leu-OMe at 22°C for 40 min. When the incubation interval was completed, 10% of foetal calf serum (FCS) was added, and the cells were washed twice with PBS. The population remaining was found to contain 6% residual monocytes as shown by flow cytofluorometry analysis.

Cells were cultured in DMEM medium (Flow Laboratories, Irvine, UK) containing 10% FCS, 200 mM glutamine, 2 g/l sodium bicarbonate, 100 µg/ml streptomycin, 100 U/ml penicillin. The proportion of viable cells before culture was determined by trypan blue exclusion. Samples containing two million BMC or LDM were incubated with 2.10^7 bacteria in a total volume of 1 ml in 24 well plates for 48 hours at 37°C in a humidified 5% CO_2 incubator. 10 µl of ConA (250 µg/ml) and 10 µl of LPS (10 µg/ml) were used as a positive control respectively for IFN-γ and for IL-1β and TNF-α. BMC and LDM were used as negative controls. 10 µg/ml of antibody (anti-CD4, anti-CD8; monoclonal antibodies for CD4 T helper, CD8 T cytotoxic/suppressor, respectively; IgG type, synthesised in mice) were added 1 hour before addition of bacteria and 24 hours after incubation. At the end of the incubation, cell-free supernatants were obtained and stored at -80°C until assayed for cytokines.

Bacterial strains

Streptococcus thermophilus (strain 158) was kindly provided by Centre de Recherche International Daniel Carasso (Plessis-Robinson, France). *Bifidobacterium*, *Lactobacillus acidophilus* and *Streptococcus lactis* and *Streptococcus casei* were obtained from Pilege (Champtoceaux, France). Before use, the bacteria were washed 3 times with DMEM medium without foetal calf serum and antibiotics.

Table 1: IL-1β, IL-2, IL-6, and IFN-γ production by BMC (blood mononuclear cells) in the presence of lactic bacteria for up to 48h

	IL-1β ng/ml	IL-2 U/ml	IL-6 ng/ml	IFN-γ U/ml
BMC	0.59±0.02	14.07±0.17	2.20±0.06	3.46±0.10
BMC + *S. thermophilus*	29.01±1.89	14.00±0.22	72.20±1.96	13.69±0.40
BMC + *Bifidobacterium*	1.02±0.02	13.84±0.24	38.92±0.54	4.14±0.33
BMC + *L. acidophilus*	0.96±0.05	14.22±0.39	46.35±0.80	40.94±1.38
BMC + *S. lactis*	1.37±0.07	14.96±0.38	56.88±1.34	48.10±1.31
BMC + *L. casei*	0.72±0.01	14.42±0.34	14.79±0.52	3.97±0.09

BMC = 2.10^6; bacteria = 2.10^7; n = 6

Cytokine assays

IFN-γ, IL-1β, IL-2, IL-6, and TNF-α were measured by specific immunoradiometric assays (Medgenix, Belgium); the sensitivities of the assays were: >5 pg/ml, >5 pg/ml, >1 U/ml, >6 U/ml and 6 pg/ml respectively. Radioactivity was measured in an automatic gamma counter (LKB-Wallac, Turku, Finland).

RESULTS

Consuming yoghurt for 15 days roughly doubled the levels of 2-5A synthetase found in the circulating BMC of 5 subjects, but had no effect on the 3 others (Figure 1), so that there was a significant increase by 38% (192±24 vs. 265±33, $p<0.05$) when considering all of them.

Streptococcus thermophilus were the only bacteria that induced the production of IL-1β. IL-2 was spontaneously produced by blood mononuclear cells, without any effect by the bacteria. In spite of this, the production IFN-γ was not produced by *Bifidobacterium* nor by *L. casei*. *L. casei* were also the less effective bacteria to induce of IL-6 (Table 1). *S. thermophilus* was able to induce the secretion of TNF-α. Mitogens (ConA and LPS), induced markedly the production of IFN-γ but not that of IL-1β and of TNF-α (Table 2). The addition of an antibody to CD4 co-receptor reduced to 1/4 the production of IFN. Interestingly, the anti CD8 had also an important effect. However, adding the two antibodies together, did not further reduce the production of IFN when compared to that observed with anti CD4 alone (Table 3).

DISCUSSION

The data presented here confirm that the ingestion of yoghurt in healthy subjects was able to increase the level of 2-5A synthetase in circulating mononu-

Table 2: IL-1β, TNF-α, and IFN-γ production by BMC in the presence of *Streptococcus thermophilus*

	IL-1β ng/ml	TNF-α ng/ml	IFN-γ U/ml
BMC	0.52±0.18	0.28±0.05	3.73±0.78
BMC + ConA	0.99±0.35	1.67±0.13	24.4±2.7
BMC + LPS	1.89±0.31	1.91±0.22	13.8±2.5
BMC + *S. thermophilus*	24.8±3.7	9.51±1.14	17.6±2.6

BMC = 2.10^6; *S. thermophilus* = 2.10^7; n = 6

clear cells. This suggests that the intestinal immune system has been stimulated by the ingestion of the non-pathogenic bacteria contained by yoghurt. The observed increase level of 2-5A synthetase suggest an augmentation of a physiological production of IFN in healthy subjects. However, the change observed here was less than the one observed previously (*Solis Pereyra* and *Lemonnier*, 1991). In our previous work, the subjects ingested in the lab a controlled yoghurt enriched in bacteria (2×10^{11}/g) once. Here the subjects were allowed to buy commercial yoghurts of their choice and to consume them at home. Thus a precise control of the quality and quantity ingested was not possible. Nor it was possible to be sure that no dairy products were consumed during the control period. Interestingly, there was a significant increase in the activity of the 2-5A synthetase, indicating that a regular ingestion of yoghurt is compatible with a maintenance of a higher level of the enzyme activity and probably with a higher production of IFN. In agreement with this, was the *in vitro* induction of the production of IFN-γ by *Streptococcus thermophilus*, one of the two bacteria contained in yoghurt.

The mechanism by which consuming of non-pathogenic bacteria could induce the production of IFN is not established. It may be suggested that these bacteria, probably their walls, rather than there cytoplasm or secreted substances (*Solis Pereyra* and *Lemonnier*, 1993), have been recognised by the immune system of the small intestine -a milieu usually poor in microorganisms- as the effect is detectable in blood four hours after feeding (*Solis Pereyra* and *Lemonnier*, 1991). Then, some lymphocytes, may be from the Peyer's patches, were liberated from the intestine and allowed to circulate in the blood as indicated by a higher level of 2-5A synthetase. A nearby mechanism might be involved to explain the spontaneous level of 2-5A synthetase observed in these subjects even then when they were not consuming fermented dairy products. Other dietary substances, like lectins from peas, have been suggested to explain this level of 2-5A synthetase (*Bocci* et al, 1988), but it is more likely that this is the result of a stimulation in the large intestine of the colon flora, as it has been shown that some of these bacteria are able to induce the production of IFN *in vitro* (*Solis Pereyra* and *Lemonnier*, 1993; *Rusch*, 1994). These data suggest that the spontaneous -thus physiological- production of IFN, indirectly detected by the spontaneous activity of the 2-5A synthetase, might be

Table 3: IFN-γ (U/ml) production by LDM (monocyte depleted lymphocyte populations) in the presence of anti-CD4 and anti-CD8 antibodies

		Anti CD4	Anti CD8	Anti CD4 + Anti CD8
LDM	1.71±0.42 (4)	–	–	2.56±0.31 (4)
LDM + *S. thermophilus*	32.2±7.9 (4)	7.83±0.74 (3)	12.2±0.90 (3)	8.18±1.67 (4)

LDM = 2.10^6; *S. thermophilus* = 2.10^7; (): number of subjects

regulated by dietary non-pathogenic bacteria fed regularly.

It is well established that activation and action of different T lymphocytes types, necessitate receptor of T cells (RCT) antigen recognition in association with major histocompatibility complex (MHC, Human Leukocyte Antigen: HLA for human) molecules (*Davis* et al., 1988). $CD4^+$ and $CD8^+$ lymphocytes interact with antigens associated with MHC I and II molecules, respectively (*Bierer* et al., 1989). Models for antigen presentation have divided antigens into two categories: endogenous (such as virus) and exogenous (such as bacteria), which are presented to T cells by class I and class II MHC respectively (*German*, 1986; *Sweetser* et al., 1989, *Morison* et al., 1986). Recently, the separation between these two pathways for presentation was called into question (*Bolonesi*, 1990). *Nuchtern* and co-authors (1990) suggest that Influenza virus proteins can associate not only with MHC I but also with MHC II molecules. The mechanism of presentation of non-pathogenic bacteria have been poorly studied. This study showed that Streptococcus thermophilus could induce IFN-γ production by monocytes depleted lymphocytes population *in vitro* via TCR-CD8-HLAI/TCR-CD4-HLAII complex, as this production was inhibited by anti-CD4 (76% inhibition) and to a lesser extent by anti-CD8 (62% inhibition).

A significant production of IFN was still existing when the antibodies, anti-CD4 and anti-CD8 were both present. This could be due to the stimulation of lymphocytes independently of TCR-CD8-HLAI/TCR-CD4-HLAII complex. This mechanism might be involved either through the cytokine network: a stimulation of other cells, such as B cells and monocytes (not tested in this study), can produce cytokines; or non protein components of *Streptococcus thermophilus* could stimulate immune cells, independently of the HLA system. It has been reported that molecules such as theichoic acid, present in the wall of Gram-positive bacteria, can induce cytokines *in vitro* (*Tufano* et al., 1991). Unfortunately, no data are available on the structure and composition including theichoic acid of the bacteria presented in this paper. All the dairy bacteria tested *in vitro* were not equally efficient to induce the production of cytokines, it is not known if this might be related in part, to differences in the composition of their membranes.

Numerous interactions are known between cytokines (*Reyes* et al., 1986). For example, IFN can stimulate the synthesis of IL-1. This was not observed, in our experiment where high levels of IFN were produced *in vitro* without any production of IL-1 (Table 1). IL-2 was spontaneously produced in vitro at the same level by BMC without any effect of the bacteria. IL-2 is con-

sidered to be necessary for a production of IFN (*Croll* and *Morris*, 1986). In our conditions this production of IFN was not related to that of IL-2, as IFN was not produced in the presence of *Bifidobacterium* and of *L. casei*.

In conclusion, non-pathogenic bacteria used in dairy products seemed to be able to stimulate the production of different cytokines *in vitro*, and also in vivo for IFN. However, the role of this IFN production is not yet clear. It is known, for example that consuming lactic bacteria renders laboratory animals more resistant to Salmonella infection (*De Simone* et al., 1988, *Hitchins* et al., 1985), but the underlying mechanism has not been established. Such a protection to infection, might be compatible with a role of the induction of the production of cytokines in a system of immune surveillance, but this has not been yet demonstrated.

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PROPHYLACTIC TREATMENT OF PIGLETS WITH LACTOBACILLUS STRAINS OF PORCINE ORIGIN

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SUMMARY

The studies presented here summarise our work directed at selecting a functional probiotic strain for oral administration to piglets. Historically the work was initiated in the mid eighties because Swedish authorities prohibited the use of low dose antibiotics in animal husbandry and alternative treatments were needed. From surveying the literature at that time, it was apparent that lactobacillus dosage could be useful, however, no single strain with conclusively proven results was reported. Our approach was to initially identify the major causative agent(s) responsible for economic losses in the pig industry. This was shown to be postweaning diarrhoea mediated by enterotoxigenic *Escherichia coli*, and especially those bearing K88 fimbriae. A strategy for *in vitro* selection was established for isolating strains of lactobacilli of host origin and which had the potential to reduce the susceptibility of the piglet to *E. coli* K88 induced diarrhoea. *In vitro* studies were directed both at identifying useful strains as well as to understand the mechanisms involved, thus allowing one to better predict the function of the strain *in vivo*. Field studies were then performed to evaluate the selected strains. It was concluded that the selection criteria used was satisfactory for isolating strains which could colonise the young piglet for extended periods, reduce the incidence of post-weaning diarrhoea and improve weight gain.

INTRODUCTION

The concept of orally administering lactic acid bacteria to piglets to improve health of the host was used as early as 1947 when *Møllgaard* (1947) dosed piglets with a lactic acid bacillus of host origin and reported improved health and improved skeletal formation. He proposed the phytic acid in ungerminated seeds in the diet interfered with absorption of calcium and phosphorus, an effect which he showed could be inhibited by lactic acid (*Møllgaard*, 1946), for example as produced by lactic acid bacteria.

The prophylactic use of lactic acid bacteria for piglets received little interest until the eighties when authorities and the consumers became concerned with the increasing need for the use of low dose antibiotics to facilitate good growth rates in intensive commercial units. Although numerous approaches for dealing with intestinal disturbances in piglets have been reported (reviewed

Table 1: Lactic acid bacteria utilised as probiotics for piglets (summarised from *Jonsson* and *Conway*, 1992).

Lactobacillus spp.	*Enterococcus faecalis*
L. acidophilus	*Enterococcus faecium*
L. lactis	
L. reuteri	*Bifidobacterium bifidus*
L. fermentum	*B. pseudolongum*
L. murinus	*B. thermophilus*

Mixed LABs prepared from the following:
L. plantarum, L. casei, L. fermentum, L. brevis, L. acidophilus, Enterococcus faecium, Streptococcus salivarius, L. delbrueckii

by *Jonsson* and *Conway*, 1992), there is increasing interest in the use of probiotics and in particular lactic acid bacteria for piglets. This is particularly evident from the number of reviews of the topic over the last decade e.g. (*Conway*, 1989; *Fuller*, 1986; *Fuller*, 1989; *Fuller*, 1992; *Jonsson*, 1985; *Jonsson* and *Conway*, 1992; *Sissons*, 1989; *Søgaard*, 1987; *Wolter* and *Henry*, 1982). As can be seen in Table 1, lactobacilli are the most commonly used lactic acid bacteria for probiotics for piglets, with *Lactobacillus acidophilus* being the most studied species. *Enterococcus faecium* has also been extensively used for piglets and a limited number of studies have reported using bifidobacteria and mixtures of several species of lactobacilli. The dose used is usually in the range of 10^9 - 10^{10} per animal per day and it is often included in the diet at a level of 10^6 - 10^7 per gram feed. A concentration of 10^9 corresponds to approximately 10 - 100 g of digesta for the adult pig and is comparable to the number of lactic acid bacteria in the stomach of the suckling pig. While the dose required for achieving demonstrable effects has been studied to a limited extent for humans and it has been suggested that at least 10^8 cells per day are required (*Gibson* and *Conway*, 1994; *Saxelin*, et al., 1991), the question has not been addressed for piglets.

The efficacy of oral administration of lactic acid bacteria to piglets is often studied in terms of the influence on (a) the stability of the digestive tract microbiota and pathogen invasion, (b) the function and morphology of the digestive tract and (c) performance and health of the pig. The most commonly studied parameter is the latter because of ease of measurement and the commercial interest in the matter. It is generally accepted today that some reports of the prophylactic use of lactic acid bacteria for piglets show no effects, while others report beneficial findings. In a few cases detrimental effects have been reported with decreased weight gain and lower feed conversion noted with dosage of an *L. reuteri* (*Ratcliffe*, et al., 1986) and in one case a higher mortality rate was noted using *Enterococcus faecium* strain M74 (*Kluber*, et al., 1985). One can hypothesis that these detrimental effects may be the result of overdosing since there has been one report that overdosing humans can have a laxative effect (*Gordon*, et al., 1957). In contrast to this one study of *Kluber* and co-workers (1985) using strain M74 , there have been at least six studies reporting improved performance with administration of strain M74 e.g. (*Moen*, 1982). Similarly, oral administration of another *L. reuteri* strain than

Table 2: Criteria used for selecting a probiotic strain for use with piglets

Host origin	
Biological activity against target	
Colonisation potential	
Survival in:	low pH, bile acids, antibiotics, additives
Stability of numbers during:	- preparation
	- storage
Stability of characteristics	

that used by *Ratcliffe* and colleagues (1986), resulted in improved mucosal morphology (*Jonsson* and *Hennings-son*, 1991). Administration of another *Enterococcus faecium* strain, referred to as C68, has resulted in less disease (*Krarup*, 1987) and improved performance (*Maeng*, et al., 1989) by some workers while others fail to show any effect (*Kornegay* and *Thomas*, 1973). This type of inconsistency has also occurred with dosage of *L. acidophilus* with some workers reporting no effect (*Kornegay*, 1985) while several other groups, e.g. *Redmond* and *Moore* (1965) note improved performance and a stabilising effect on the microbiota. Several influencing factors for the inconsistent findings have been presented (*Conway*, 1989; *Fuller*, 1986; *Fuller*, 1989; *Fuller*, 1992; *Jonsson*, 1985; *Jonsson* and *Conway*, 1992; *Sissons*, 1989; *Søgaard*, 1987; *Wolter* and *Henry*, 1982), namely, the age of the animal, environmental conditions, sensitivity of the gut to disturbances, diet as well as strain variation and variation in the viability of the preparation.

The US markets for animal probiotics have increased about 5-fold over the last decade and this largely reflects the new approaches to probiotics. It can be said that workers have identified the problems associated with probiotics of yesteryear and that there is new hope in the future with the appearance of better strains which are biologically targeted for specific applications.

The work presented here was initiated almost a decade ago when the Swedish government introduced legislation prohibiting the use of low-dose antibiotics in animal feeds and alternative methods were required for the pig industry. On surveying the literature, it appeared that the oral administration of lactic acid bacteria held promise, but that there was no single strain which was clearly significantly better and most strains used at that time failed to produce beneficial effects under some condition. The work presented here can be seen as a case study in which the target was identified and a criteria for selection of a desirable probiotic strain prepared. The mechanisms of action of the selected strains were studied *in vitro* and finally field trials in a commercial piggery were performed.

METHODOLOGY

The strategy used for obtaining a functional probiotic strain for piglets was also followed when selecting a probiotic strain for human usage as included in a recent review (*Conway* and *Henriksson*, 1994). Basically the approach has been to identify the target for the probiotic and then establish a strain

selection criteria as presented in Table 2. Once specific strains were identified, the mechanisms of action was studied *in vitro* and ultimately, *in vivo* field trials for efficacy conducted.

In order to isolate a lactobacillus which could be beneficial to piglets, we initially focused on the causative agents for mortality and economic loss in commercial pig raising establishments. Bacterial induced diarrhoea was the largest single contributor to mortality rates and the agent was predominantly enterotoxigenic *Escherichia coli,* with those bearing K88 fimbriae being particularly frequent (*Jonsson* and *Conway*, 1992). Consequently, when using the selection criteria outlined in Table 2 (*Conway* and *Henriksson*, 1994; *Gibson* and *Conway*, 1994), potential strains were screened for biological activity against enterotoxigenic *E. coli* bearing K88 fimbriae. This was measured in terms of inhibition of growth and adhesion of the pathogen. Growth inhibition was assessed by measuring zones of inhibition around a colony on an agar plate (*Conway*, to be submitted), and also by monitoring the growth of the pathogen spectrophotometrically in media containing spent culture supernatant of the isolates mixed with fresh growth media (50:50) (*Rojas* et al., to be submitted). Inhibition of adhesion of the pathogen was investigated using dialysed and fractionated spent culture in an *in vitro* adhesion assay as previously described (*Blomberg*, et al., 1993).

Furthermore since *E. coli* K88 colonise the distal ileum, lactobacillus isolates from the piglet digestive tract were selected for the capacity to colonise the stomach and hence be continually seeded into the distal ileum as viable cells. The diversity of lactobacilli isolated from the various regions of the gut were studied by comparison of the SDS-PAGE protein profiles of lysozyme treated cells.

Colonisation capacity was evaluated by testing the adhesive nature of the isolates in an *in vitro* adhesion assay using radioactively labelled bacteria and epithelial mucosa (*Henriksson*, et al., 1991), bearing in mind the limits of this type of assay and with strict attention to the correct controls necessary for interpreting the data (*Conway* and *Henriksson*, 1994). Survival of the isolates in low pH, bile acids and enzymes was evaluated by appropriate additions to buffered saline and growth media as previously reported (*Conway* and *Henriksson*, 1994; *Gibson* and *Conway*, 1994). The stability of viability was monitored in both the initial material as well as after storage at a range of temperatures by measuring the colony forming units. Even though viability may be maintained over time, we felt the need to also ensure that the characteristics of the strain did not alter and hence when the viability was monitored, so too were the characteristics such as survival, adhesion and effects on pathogens (*Conway* and *Henriksson*, 1994) .

In the first stage of *in vivo* trials (*Conway* and *Rönnow*, to be submitted), piglets were orally dosed with an erythrosine solution immediately prior to oral administration of a preparation of *Lactobacillus murinus* strain C39 suspended in a solution of infant formula. Animals were dosed twice, once in the evening and again next morning, with the *L. murinus* preparation during the first two weeks of life. Rectal swabs were then collected weekly for a 9 week period after which time some piglets from each group were sacrificed. Samples were analysed for the number of colony forming units (CFU) of lactobacilli and coliforms and the ratio of the two calculated for each sample. From the lactobacilli cultured, isolates were randomly picked for studying the pres-

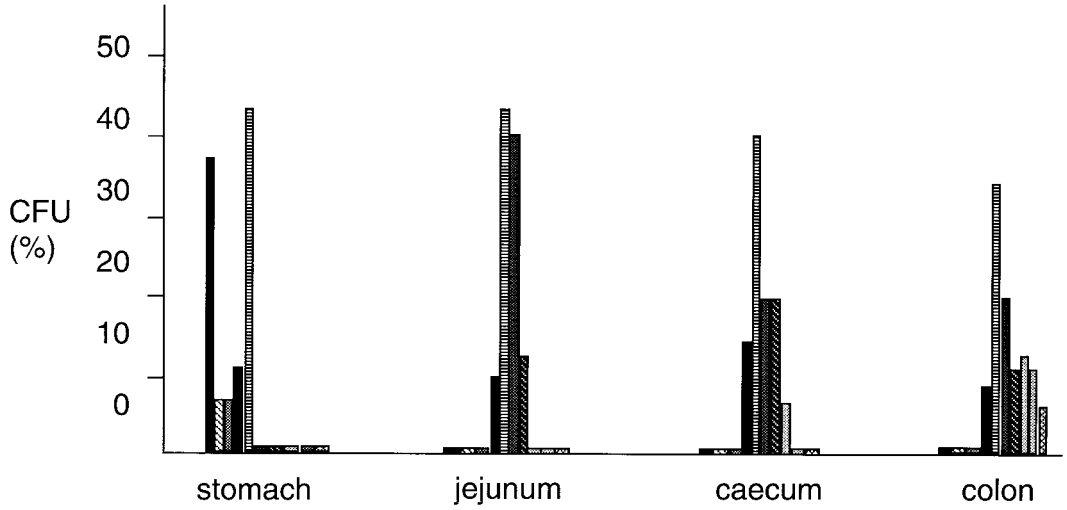

Figure 1: Diversity of lactobacilli in the porcine gastrointestinal tract. Protein profiles, as visualised by SDS-PAGE, of lactobacilli from the various regions of the piglet digestive tract were grouped according to the extent of similarity. Bars shaded in the same pattern represent isolates with similar profiles. The results are presented as the percentage of the viable isolates (colony forming units, i.e. CFU) from each region, which had the particular profile (*Henriksson* et al., 1995).

ence of the administered strain both in rectal samples and throughout the gut of the sacrificed animals. The *L. murinus* strain was positively identified immunologically and morphologically.

In a second field trial (*Conway*, to be submitted), piglets in a commercial piggery were given *ad libitum* access to the *L. fermentum* strain via the creep feed and the weaning feed since the strain was added as a freeze dried supplement to the powdered feed. Erythrosine was orally dosed to the piglets immediately prior to weaning and this was followed by an increased dose of the *L. fermentum* strain. The incidence of diarrhoea and weight gain over the 9 week period were monitored in the negative (no additions to the feeds) and positive (antibiotics added to feeds) control animal and the test piglets.

RESULTS AND DISCUSSION

Reviews of published studies show a positive trend that piglet performance and health may be improved by prophylactic administration of lactic acid bacteria (*Conway*, 1989; *Fuller*, 1986; *Fuller*, 1989; *Fuller*, 1992; *Jonsson*, 1985; *Jonsson* and *Conway*, 1992; *Sissons*, 1989; *Søgaard*, 1987; *Wolter* and *Henry*, 1982), however, conflicting results have been reported and it is generally agreed that more attention to strain selection could yield more consistent findings (*Conway*, 1989; *Havenaar*, et al., 1992). The work presented here summarises our approach used for developing a functional lactobacillus preparation for prophylactic use in piglets. Since a major cause of economic loss for the pig industry is enterotoxigenic *E. coli* K88 induced diar-

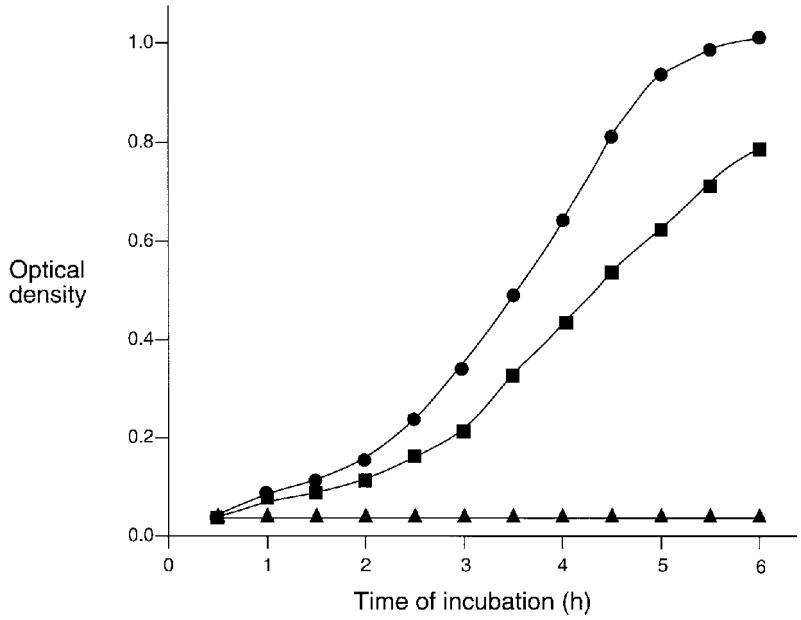

Figure 2: Growth of *Escherichia coli* K88 in brain heart infusion broth (BHI) (circles), BHI + spent culture supernatant of *Lactobacillus fermentum* strain 104 (triangles) or in BHI + dialysed spent culture supernatant of *L. fermentum* strain 104 (squares). Results are expressed as the increase in optical density (590 nm) over 6 hours (*Conway*, to be submitted).

rhoea (*Jonsson* and *Conway*, 1992), the strain selection criteria targeted this pathogen. Consequently, lactobacillus isolates from the porcine digestive tract were screened using the selection criteria outlined in Table 2. In addition, emphasis was placed on selecting strains which could colonise the pars oesophageal region of the stomach since this region is densely colonised by lactobacilli and as epithelial cells are sloughed off from the mucosa, associating lactobacillus cells are carried into the lumenal contents. This process allows a continual inoculum of the lactobacilli to the small intestine which is the site of colonisation of *E. coli* cells bearing K88 fimbriae. An *in vitro* adhesion assay using pars oesophageal mucosa was used to screen for the potential of the isolates to colonise this tissue. Assuming the lactobacillus cells would be more effective if metabolically active in the small intestine, an attempt was made to select strains of host origin with this capacity. Isolates were selected which had the capacity to survive the rigours of the tract e.g. low pH, bile acids, intestinal enzymes, low nutrients.

The data presented in Figure 1 groups, according to the SDS-PAGE protein profiles of lysozyme-treated cells, lactobacillus isolates from various regions of the gastrointestinal tract. From these data, it is apparent that some groups of lactobacilli were localised in the upper regions of the tract, while others were only found in the lower regions. One profile (histogram with shaded horizontal lines, Figure 1) was detected throughout the tract and an isolate consistent with this profile should therefore have a greater chance of colonising the entire tract (*Henriksson* and *Conway*, 1995).

Biologically activity was initially screened by overlaying the pathogenic *E. coli* K88 strain on colonies of the

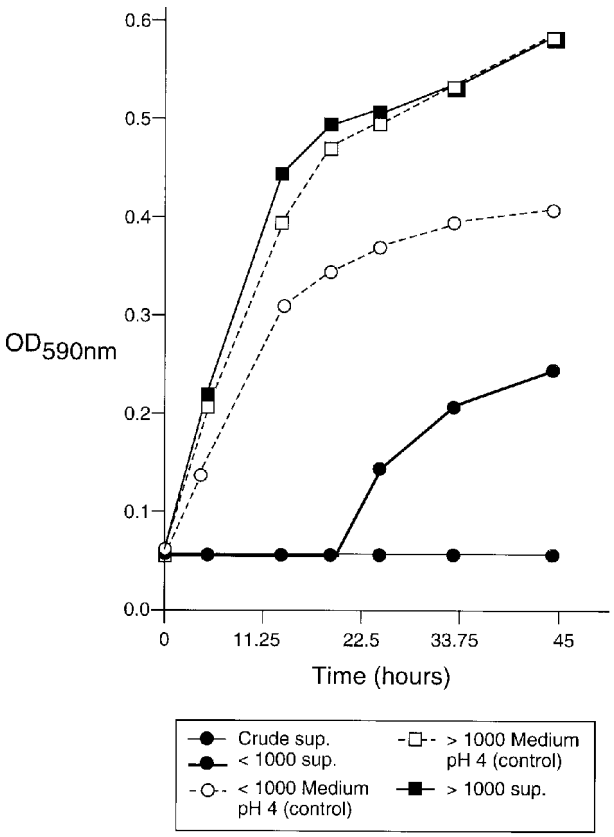

Figure 3: Growth of *Escherichia coli* K88 in modified brain heart infusion broth (MBHI) containing: a 1:1 dilution with spent culture supernatant of *L. fermentum* 104, or culture supernatant dialysate (<1000 Mr), or MBHI broth dialysate (<1000 Mr) adjusted to yield pH 4, or MBHI broth retentate (>1000 Mr) at pH 4, as well as retentate of MBHI spent culture supernatant. Results are presented as the optical density (590 nm) of the growth media (*Rojas* et al, to be submitted).

lactobacillus isolates. Zones of inhibition of growth varied from nothing to 35 mm in diameter for the strains studied. Some isolates producing large zones were further screened for the presence of growth inhibitors in the spent culture supernatant. An isolate identified as *Lactobacillus fermentum* was shown to produce spent culture supernatant that totally inhibited the growth of *E. coli* K88 when combined with fresh culture medium (Figure 2). It was further shown that the retentate after dialysis (membrane cut-off 8-10 K) as had some growth inhibitory activity (Figure 2). This activity was destroyed by protease and heat treatment, however, was not stable with time. Even primary cultures stored at -70°C for 2 years did not retain the activity and one can suggest that the activity may be restored with animal passage as can occur for virulence factors not expressed on subculturing. It has subsequently been shown that the lower molecular weight components (<1000 Mr) were also bacteriostatic and that this effect was enhanced when the smaller molecular weight components were included with the higher molecular weight (>1000) components (Figure 3). This activity was pH sensitive and one can question

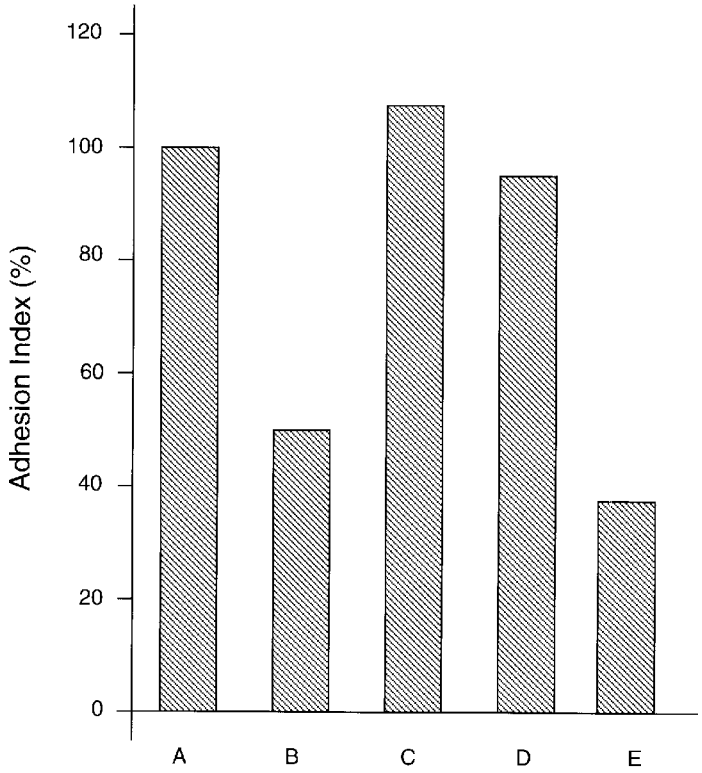

Figure 4: Adhesion of *Escherichia coli* K88 fimbriated cells to immobilised porcine ileal mucus in the presence of buffer (A; control), *Lactobacillus fermentum* strain 104 spent culture fluid (B) and fractionated spent culture fluid of strain 104 yielding up to 10 K (C), 10-30 K (D) and >30 K (E) relative molecular weight. Results are expressed as a percentage of the adhesion obtained for the control (A) (*Blomberg* et al, 1993; *Conway* unpublished data).

whether this activity functions in the gut.

The potential for the lactobacillus cells to protect the host from pathogen invasion can also be discussed in terms of the effects of lactobacillus metabolites on adhesion of the pathogen to the epithelial mucosa, since it has been established that this is a pre-requisite for colonisation by the pathogen. Using an *in vitro* adhesion assay, it has been established that the presence of spent culture supernatant of the lactobacilli of porcine origin inhibits the adhesion of the *E. coli* K88 fimbriated cells (Figure 4). In this study (*Blomberg* et al., 1993), it was also shown that the interference was demonstrable if the mucosal surface, but not the bacterial surface, was treated with the spent culture supernatant prior to the assay. Furthermore fractionation of the supernatant revealed that the activity was detectable in the retentate with an Mr of greater than 30 K. It was concluded that component(s) interfered with the interaction between the K88 fimbriae and the mucosal receptor and the mechanism is undergoing further investigation.

Two strains, identified as *L. murinus* and *L. fermentum*, showed inhibition of growth of the *E. coli* K88 cells in the primary screen using the overlay technique (*Conway*, to be submitted), and also adhered well to non-secreting stomach tissue in the *in vitro* adhesion

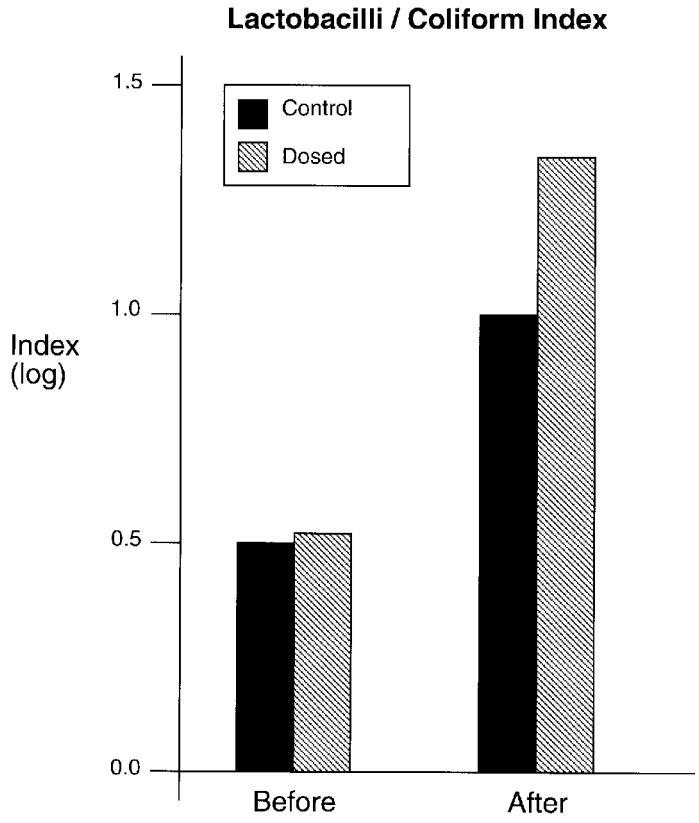

Figure 5: Ratio of lactobacilli to coliforms in the faeces of piglets before and after dosing with *Lactobacillus murinus*. Results are presented as the ratio of the colony forming units (CFU) of lactobacilli to CFU coliforms using log CFU per g faeces (*Conway* and *Rönnow*, to be submitted).

assay (*Henriksson* and *Conway*, 1992; *Henriksson*, et al., 1991). These strains survived low pH, bile acids and nutrient deprivation (data not presented), and were investigated in more detail. When *L. murinus* was sub-cultured in laboratory media, some decrease in adhesive capacity was noted. In contrast, *L. fermentum* exhibited stable adhesive characteristics. When cell wall extracts were reductively methylated, a carbohydrate with affinity for the porcine gastric squamous tissue could be identified (*Henriksson* and *Conway*, 1992). This carbohydrate did not adhere to gastric mucus from the secreting region and it was suggested that proteins could be involved in association of the strain with mucus (*Henriksson*, 1993). From the data, one can hypothesise that *L. murinus* and *L. fermentum* have the potential to colonise the digestive tract of piglets and be biologically active against *E. coli* K88 bearing strains.

In the first stage of the *in vivo* trials, piglets were dosed with *L. murinus* during the first 2 weeks of life and the lactobacilli and coliforms in rectal swabs were quantified. As can be seen in Figure 5, the lactobacillus:coliform ratio was greater after oral dosage. The lactobacilli cultured from the rectal samples, as well as isolates from the lumenal contents and mucosal homogenates of sacrificed 9 week old piglets, were subcultured and immuno-

Table 3: The weight gain recorded for piglets orally dosed with lactobacillus

Group	Weight increase of test group (%)		
	0-37 d	0-63 d	37-63 d
Negative control	35*	72*	55*
Positive control	32	28	4

* Significantly different from control, $p<0.05$
No. of litters per group = 8

Results are expressed as the percentage weight gain in the test group relative to that noted for the negative and positive controls, the latter group receiving antibiotics. Piglets were weaned at 37 days and the results are presented for the entire period of study (0-63 days), pre-weaning (0-37 days) and post-weaning (37-63 days) (*Conway*, to be submitted).

logically screened for the presence of the introduced *L. murinus*. This strain was regularly detected in the rectal swabs for the 9 weeks of the study and in lumenal and mucosal samples from the 9 week old pigs (*Conway* and *Rönnow*, to be submitted). It was concluded that the criteria used for strain selection yielded a strain with the capacity to colonise the piglet digestive tract for extended periods of time.

In the second stage of the field trials, the *L. fermentum* strain was orally dosed to piglets in a commercial piggery. In this study the parameters investigated include the effects on the incidence of diarrhoea and on the weight gain over a 9 week period. As can be seen in Table 3, the piglets receiving the *L. fermentum* (test group) significantly gained more weight then did piglets in the negative control group which received no supplements and also gained more weight than did those in the positive control group receiving an antibiotic supplement. Consistently, no post-weaning diarrhoea was detected in the litters receiving the *L. fermentum*, while 80% of the negative control litters suffered diarrhoea (Table 4). It was concluded that the criteria used for strain selection (Table 2), yielded isolates which had the capacity to colonise the digestive tract and the results provide indirect evidence that the strains also exerted biological activity on the pathogenic *E. coli* K88 when orally dosed.

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Table 4: The incidence of diarrhoea in piglets before and after weaning at 37 days for negative and positive controls (receiving antibiotics) as well the test group being orally dosed with lactobacilli

Group	No. of litters	Before weaning	After weaning
Neg. control	15	47 %	80 %
Pos. control	15	33 %	27 %
Test	8	25 %	0 %

Results are expressed as the number of sows with diarrhoea in the litter as a percentage of the total number of sows.

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MICROBIAL FEED ADDITIVES FOR RUMINANTS

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SUMMARY

Microbial feed additives are used in ruminant feeds for three different purposes. The first is for the same reason that probiotics are used in non-ruminants, namely stabilisation of the intestinal flora; this is applicable only in young, pre-ruminant animals, however, where lactobacilli, enterococci and yeast have been reported to be helpful in preventing diarrhoea and in improving live weight gain in calves and lambs. The second aim in young animals is to enhance the development of the adult rumen microflora, because this stimulates development of rumen structure and accelerates weaning. The same organisms and *Aspergillus oryzae* fermentation extract appear to achieve this objective, and inoculations with rumen microorganisms have also been found to be effective experimentally. In the adult ruminant, only yeast and fungal cultures have been widely reported to be effective. Responses in meat and milk production are highly variable, with an average response of 7 to 8% in each case to both products. Increased feed intake usually appears to drive the response. In turn, improved intake occurs because of a more rapid breakdown of fibre in the rumen, and an enhanced microbial protein flow from the rumen has been reported. Both types of additive cause a marked increase in the viable count of anaerobes recovered from rumen fluid, but for different reasons. Yeast appears to function by removing traces of oxygen that may be toxic to rumen bacteria: non-respiratory yeasts did not stimulate numbers. In contrast, the mode of action of *A. oryzae* appears to be associated with its enzyme activity. Neither yeast nor *A. oryzae* grows to a significant extent in the rumen.

INTRODUCTION

The digestive anatomy and physiology of ruminants is markedly different to that of monogastric animals, including pigs and man. Microbial feed additives therefore have several objectives quite different to those used with non-ruminants. During early life, when milk is the main ingredient of the diet, the rumen tissue structure is undeveloped (Figure 1), and food tends to bypass the rumen. The first function of microbial feed additives during neonatal life, the prevention of diarrhoea by modifying the flora of the small intestine, in calves and lambs is essentially the same problem as in other species and the microbial feed additives can be regarded as *probiotics* in the same way. In young ruminants, however, commercial benefits can also be obtained by enhancing the rate at which the rumen flora and fauna develop and thereby accelerating the

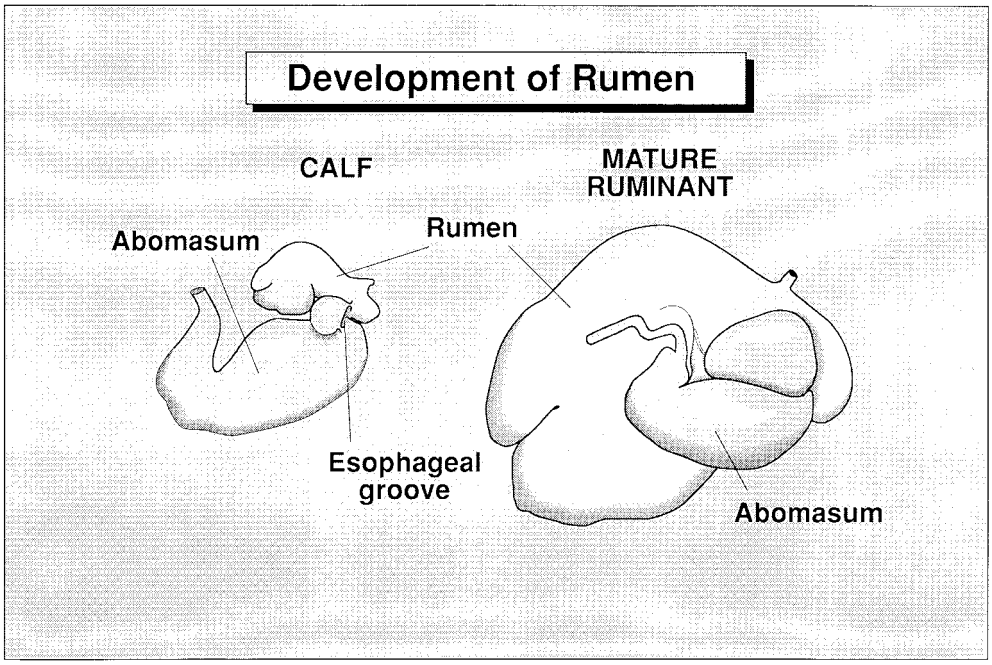

Figure 1: Development of structure of the foregut in ruminants. In the newborn calf, the rumen is rudimentary, and milk bypasses the rumen, passing through the oesophageal groove direct to the omasum. It then passes to the abomasum, which is the gastric stomach of ruminants. In the adult animal, the rumen is much more developed, and food is retained for 12 to 36 h. The large anaerobic microbial population of the rumen only develops to its full potential when solid food is consumed and the rumen structure is well-developed.

onset of weaning. Microbial additives can contribute to this development. Finally, once the fermentation has fully established in the adult animal, the considerations change once more. Enhancing fibre breakdown and stabilising the fermentation then become uppermost in importance. Microbial feed additives can benefit all three objectives in ruminants, but the type of additive best suited for the production situation will depend on the objective to be fulfilled.

PRE-RUMINANTS: PREVENTION OF DIARRHOEA

Diarrhoea caused by enterotoxigenic bacteria colonising the gut represents a serious economic constraint on the rearing of young animals. *Massip* and *Pondant* (1975) reported that up to 6.5% of Belgian calves died due to intestinal disorders during the first month of life, while less severe cases of infection might reduce intestinal nutrient absorption and animal performance (*Youanes* and *Herdt*, 1987). Increased coliform counts have been reported in the intestine of calves suffering from diarrhoea (*Smith*, 1971; *Younas* and *Herdt*, 1987). *Guard* (1986) states that *Escherichia coli* tends to cause diarrhoea mainly in young animals (<1 week of age) while increased coliform counts

Table 1: Effects of microbial feed additives in pre-ruminants

Microbial species	Animal	Observed effects	References
Lactobacillus spp.	calves	decreased coliform count	Ellinger et al., 1978 Bruce et al., 1979 Gilliland et al., 1980
	calves	reduced scouring	Bechman et al., 1977 Beeman, 1985 Bonaldi et al., 1986
	calves	improved feed intake/ liveweight gain	Wren, 1987 Lee and Botts, 1988
	lambs	lower mortality	Pond and Goode, 1985
	lambs	improved feed intake/ liveweight gain	Umburger et al., 1989
Streptococcus faecium	calves	improved feed intake/ reduced scouring	Hefel, 1980 Ozawa et al., 1983 Maeng et al., 1987 Svozil et al., 1987 Tournut, 1989
Saccharomyces cerevisiae	calves	improved feed intake/ liveweight gain	Fallon and Harte, 1987 hughes, 1988
	lambs	improved feed intake/ liveweight gain	Wells and Mason, 1976 Jordan and Johnston, 1990
	calves	decreased effects of transport stress	Phillips and von Tungeln, 1985
Aspergillus oryzyae	calves	improved feed intake/ liveweight gain	Allison and McCraw, 1989 Beharka et al., 1991

were also noticeable around the time of weaning (*Karney* et al., 1986). To induce diarrhoea by enterotoxin production, *E. coli* must first colonise the gut (*Guard*, 1986). It has been suggested that probiotics might be used either to displace enterotoxigenic *E. coli* from the gut wall or to promote a healthy bacterial population which exclude coliforms from the gut (*Fuller*, 1989). Some instances of their success have been recorded (Table 1).

Lactobacillus acidophilus decreased coliform numbers in the intestine of calves (*Ellinger* et al., 1978; *Bruce* et al., 1979). *Gilliland* et al. (1980) noted that *L. acidophilus* strains originally isolated from calves were more effective in this respect than those isolated from pigs. *L. acidophilus* alone or in combination with other lactobacilli has been reported to reduce scouring and increase liveweight gain in calves in some (*Bechman* et al., 1977; *Beeman*, 1985; *Bonaldi* et al., 1986) but not all trials (*Jonsson*, 1985; *Jonsson* and *Olsson*, 1985). *Lactobacillus* mixtures have also been effective in reducing mortality in wean-stressed lambs (*Pond* and *Goode*, 1985). *Aldrovandi* et al. (1984) and *Wolter* et al. (1987) both noted that live lactobacilli are more effective than dead cells. Other bacteria have also been used. *Streptococcus faecium* has been

reported to reduce scouring and improve weight gain between birth and weaning (*Hefel*, 1980; *Maeng* et al., 1987; *Svozil* et al., 1987; *Tournut*, 1989). *Tournut* (1989) reported that a mixture of *L. acidophilus* and *S. faecium* reduced the incidence of diarrhoea by almost 70% and mortality by 99% when fed to calves between birth and 5 days of age. *Bacillus acidophilus* and *Bacillus toyi* have both been reported to reduce scouring in young calves (*Hatch* et al., 1973; *Tournut*, 1989).

Although the above results demonstrate that lactobacilli and streptococci can reduce diarrhoea in young ruminants, their mode of action remains elusive. As noted above, lactobacilli have been shown to prevent coliform colonisation of the gut in calves (*Ellinger* et al., 1978; *Bruce* et al., 1979), while streptococci have been shown to prevent coliform proliferation in the intestines of non-ruminant species (*Underdahl* et al., 1982; *Wadström*, 1984). Several explanations have been put forward to explain the effect of lactobacilli and streptococci on *E. coli* in the gut. Adhesion to the gut wall may prevent colonisation by coliforms (*Muralidhara* et al., 1977; *Barrow* et al., 1980). Alternatively, these bacteria may in some way neutralise enterotoxin. Lactobacilli have been shown to produce an as yet unidentified metabolite capable of neutralising *E. coli* enterotoxin in pigs (*Mitchell* and *Kenworthy*, 1976). They may produce organic acids and thereby reduce gastric pH. Acid conditions inhibit the growth of *E. coli in vitro*. As many strains of lactobacilli and streptococci produce large quantities of lactic acid *in vitro* (*Holdeman* et al., 1977), it has been suggested that they might reduce intestinal pH and thus reduce *E. coli* overgrowth (*Fox*, 1988). Probiotic strains may possess bactericidal activity. Lactobacilli have been reported to produce hydrogen peroxide, which is bactericidal *in vitro* (*Reiter* et al., 1980). *L. lactis* stimulated the lactoperoxidase thiocyanate system in the intestines of calves (*Reiter*, 1978), which reduced the ability of *E. coli* to survive in the gut (*Reiter* et al., 1980). *E. coli* colonised the gut if reducing agents were used to reverse the effect of lactoperoxidase thiocyanate (*Reiter* et al., 1980). *Newman* et al. (1990) identified a heat-stable, >5,000 Da factor produced by *Enterococcus faecium* which was capable of inhibiting the growth of *E. coli*, *Enterococcus faecalis* and other related bacteria. However, the importance of such substances *in vivo* remains unclear (*Fuller*, 1989).

PRE-RUMINANTS: ACCELERATION OF RUMEN DEVELOPMENT

In young ruminants, the distance between the end of the oesophagus and the reticulo-omasal orifice, through which food leaves the rumen, is small (Figure 1). Young animals have a reflex which closes the vestigial oesophagus between these two orifices, the so-called oesophageal groove, so that food passes directly from the oesophagus to the omasum and then to the abomasum. Some milk inevitably spills into the rumen, however, and provides substrates for the growth of microorganisms. Like other young mammals (*Savage*, 1977), ruminants are born with a sterile gastrointestinal tract (*Cushnie* et al., 1981). However, bacterial colonisation is rapid, with *E. coli* detectable in all areas of the digestive tract of lambs and calves 8 h after birth, and lactobacilli and streptococci detectable from 24 h onwards (*Smith*, 1965). In healthy animals, lactobacilli quickly colonise the gut, displacing coliforms and reaching

populations of 10^7-10^9/g throughout the intestines by 1 week of age (*Smith*, 1965; *Karney* et al., 1986). Ample evidence now exists that most of the strict anaerobes that become predominant in the adult rumen, even methanogens, are already present in the rumen one or two days after birth (*Ziolecki* and *Briggs*, 1961; *Fonty* et al., 1987; *Morvan* et al., 1994). One aim of microbial feed additives is therefore to enhance the growth of these organisms and to establish as rapidly as possible a healthy, fibre-digesting fermentation in the rumen.

As the animal begins to consume solid feed, the microbial population in the rumen increases and begins to resemble that of the adult ruminant (*Fonty* et al., 1987; *Dehority* and *Orpin*, 1988). The end products of microbial fermentation encourage the development and extension of the rumen (*Warner* et al., 1953, 1955), such that around the time of weaning the rumen is fully developed both as a digestive and absorptive organ (*Thivend* et al., 1979). Rapid development of the rumen and a successful transition from liquid to solid feed is of great importance in the profitability of modern stock rearing operations, both in terms of reduced labour and feed costs and because digestive disorders are less frequent in weaned as opposed to liquid-fed calves (*van Horn* et al., 1976; *James* et al., 1984; *Roy*, 1980).

Although there is good evidence that microbial feed additives can be beneficial to calves without necessarily having any influence on the prevalence of diarrhoea (Table 1), little microbiology has been done to determine the mode of action, particularly in terms of the rumen. *Lactobacillus* spp. are generally considered to be incompatible with the adult rumen flora: they produce lactic acid, which is problematic in the maintenance of a stable rumen pH (*Slyter*, 1976). Activity of enterococci against pathogens in the rumen may occur, but is not documented. It is more likely that the effects of these bacterial genera are post-ruminal.

Probiotic inocula containing rumen organisms may be useful in promoting the development of an adult flora, but these have only so far been used experimentally. *Theodorou* et al. (1990) reported that a probiotic based on an anaerobic rumen fungus (*Neocallimastix* sp.) increased intake and live-weight gain in calves following weaning, while *Ziolecka* et al. (1984) and *Ziolecki* et al. (1984) reported that a stabilised rumen extract enhanced liveweight gain and stimulated rumen development in calves during weaning.

Products based on yeasts or aerobic fungi are used in young as well as adult ruminants. Their effectiveness is summarised in Table 1. *Beharka* et al. (1991) found that *A. oryzae* extract stimulated dry matter intake in calves and allowed them to be weaned earlier. Rumen development was stimulated by *A. oryzae*, with higher counts of total, amylolytic, pectinolytic, cellulolytic and hemicellulolytic bacteria from week 2 of life onwards. A fungal extract supplemented with *Streptoccocus bovis* stimulated bacterial numbers in the rumen of calves over the first 30 days after birth (*Kmet* et al., 1988). A similar preparation stimulated rumen fermentation in newly weaned lambs (*Bara* and *Kmet*, 1987). The mode of action of fungal extracts in pre-ruminants can only be speculated at present, but it is reasonable to suggest that they may involve the removal of O_2, which is inhibitory to strict anaerobes, or that they contain enzymes that enhance fibre digestion by the indigenous flora, as occurs in adult ruminants.

ADULT RUMINANTS: PAST AND PRESENT USE OF MICROBIAL FEED ADDITIVES

Yeast and yeast-containing by-products have been used in ruminant diets for many years. In 1925 *Eckles* and *Williams* published a report on the use of yeast as a supplementary feed for lactating cows and brewer's yeast has been successfully used as a protein source in ruminant diets (*Carter* and *Phillips*, 1944; *Johnson* and *Remillard*, 1983). The application of low levels of yeast (<1% of dietary DM) to dairy cow diets first received attention in the 1940's and 50's. *Renz* (1954) reported that the inclusion of 50 g/d of an active yeast increased milk yield by 1.1 kg/d. *Beeson* and *Perry* (1952) reported a 6% increase in the daily gain of steers fed 8 g/d of active dried yeast. However, results were variable with many studies reporting little or no increase in production (*Norton*, 1945; *Renz* and *Koch*, 1956; *Lassiter* et al., 1958). Publications dealing with *A. oryzae* are much more recent, beginning in the mid-1980's (*Harris* et al., 1983; *van Horn* et al., 1984; *Huber* et al., 1985), and again there seems to be a variability in response (*Newbold*, 1990). Where responses to these fungal feed additives occur, they appear to improve the nutrition of growing or adult ruminants much more than would be expected from their gross nutrient composition.

ADULT RUMINANTS: FUNGAL FEED ADDITIVES

The products with which we are most familiar are Yea-sacc (based on *S. cerevisiae*; Alltech Inc., Nicholasville, KY 40356, USA), Diamond V Yeast Culture (also *S. cerevisiae*; Diamond V Mills, Cedar Rapids, IA 52407, USA), and Amaferm (*A. flavus-oryzae;* BioZyme Enterprises Inc., St. Joseph, MO 65404, USA). Increasing numbers of products are becoming available internationally. There is no reason to suppose that these are not effective, especially if production data are available. Equally, as is described below, not all yeast or *A. oryzae* preparations produce the same effects on fermentation as others, and therefore not all yeasts or fungi would be expected to have similar nutritional effects.

Yeast products are supplied as mixtures of live and dead yeast cells together with an element of the medium in which the yeast was grown, or distillers dried solubles. Because the medium component is claimed to be important in the products' activity, the accepted terminology for the supplement is "yeast culture" (YC) rather than simply yeast (AAFCO, 1986).

A. oryzae fermentation extract (AO), on the other hand, consists of fungal spores and mycelium dried on to a base of wheat bran. The viability of the preparations appears to be quite different. Yeast culture has a viability of 10^9-10^{10} live cells/g (*Dawson* et al., 1990) or 2 x 10^7 live cells/g (*C.W. Stone*, personal communication) depending on the product, whereas *A. oryzae* fermentation extract contained 1.6 x 10^3 viable cells/g (*Newbold* et al., 1991). Fungal feed additives can be used either by sprinkling on the feed or by incorporation into a compound diet. Experiments have also been done where *A. oryzae* was administered as an inoculant to silage (*Harris* et al., 1983).

ADULT RUMINANTS: MILK AND MEAT PRODUCTION RESPONSES TO FUNGAL ADDITIVES

The general pattern with ruminants receiving fungal feed additives is that production, whether of meat or milk, is improved. *Williams* and *Newbold* (1990) reviewed this area, and they noted that 8 trials with AO produced an average 4.3% improvement in milk yield. A similar analysis of 9 YC trials resulted in an average improvement of 5.1%. These averages were calculated from ranges of 91.0-112.0% for AO and 96.3-116.7% for YC, and they may therefore conceal an even better response under optimum dietary or nutritional circumstances. More recently, *Wallace* and *Newbold* (1993) summarised results from 18 lactation studies with yeast and concluded that the response ranged from a 6.8% decrease to a 17.4 % increase in milk yield. The average value was 7.8%. Latest trials continue to reflect the variability in response to fungal additives. *Smith* et al. (1993) found no effect of YC in dairy cows receiving three corn silage/alfalfa hay diets, yet *Piva* et al. (1993) found increases in total and fat-corrected milk production of 3.1 and 9.3% respectively in response to YC. AO gave an increase in milk protein content of 2% in a commercial dairy herd (*Higginbotham* et al., 1993), but *Sievert* and *Shaver* (1993) observed no response in a smaller trial.

Less information is available for growing ruminants than lactating animals. Improved liveweight gain has, like milk production, been observed in some studies but not in others. *Adams* et al. (1981) found that steers had an improved daily weight gain of 1.39 kg with YC compared with 1.34 kg in controls. As with many responses of this magnitude, the increase did not reach statistical significance. *Edwards* et al. (1990) found no significant improvement with YC in liveweight gain from 135 kg to slaughter, although once more the trend was favourable. The opposite trend was observed by *Deaville* and *Galbraith* (1990) with Angora goats. Beef cows and calves fed a poor quality pasture improved weight gain from 0.57 to 0.80 kg/day with AO (*Wiedmeier*, 1989). *Mir* and *Mir* (1994) reported an improved feed utilisation efficiency in the first year with steers, but no other significant positive effects.

A crucial feature of the effectiveness of YC and AO seems to be the diet and the nutritional demands of the animal. *Williams* et al. (1991) demonstrated how sensitive the effects of YC can be to a relatively small change in dietary composition. A milk yield response of 4.1 kg/day to YC added to a 40:60 hay:concentrate diet fell to zero when the ratio was 50:50; the milk yield response occurred with hay as the forage but not with ammonia-treated straw. *Gomez-Alarcon* (1988) found similar interactions with forage:concentrate ratio for AO in cows, and *Huber* et al. (1985) observed that an AO supplement increased milk production of cows fed normal but not high forage diets. In contrast, intake responses of 26% occurred when AO was administered via the rumen cannula to steers grazing cool-season grasses (*Caton* et al., 1993); the response varied according to the maturity of the grass. Much work remains to be done to delineate these relations for different diets.

The response to fungal feed additives, as with any feed additive such as protein supplements, must depend on animal requirements and management, as pointed out clearly by *Chase* (1989). Thus cows in early lactation responded better to YC than those in later stages (*Harris* and *Lobo*, 1988; *Günther*,

1989). Similarly, the response to AO is greatest in early as opposed to mid or late lactation (*Wallentine* et al., 1986; *Kellems* et al., 1987). These nutritional effects will complicate the investigation of dietary interaction, and it would help if the precise modes of action of YC and AO were known.

ADULT RUMINANTS: EFFECTS ON INTAKE AND DIGESTION

Most studies indicate that, where a response is observed, fungal feed additives increase feed intake rather than alter feed conversion efficiency (*Adams* et al., 1981; *van Horn* et al., 1984; *Malcolm* and *Kiesling*, 1986; *Harris* and *Lobo*, 1988; *Edwards* et al., 1990; *Gomez-Alarcon* et al., 1990; *Williams* et al., 1991). Only occasionally is improved feed efficiency a possible benefit (*Günther*, 1989). *Williams* and *Newbold* (1990) calculated that the improvement in the intake of dairy cows corresponded well with the observed effects on productivity. The main effects of fungal feed additives are therefore regarded as being intake-driven. Many factors are known to influence appetite, but the ones that have been considered for YC and AO in ruminants have been palatability, the rate of fibre digestion (thus directly affecting gut fill), the rate of digesta flow, and protein status.

Yeast extracts and *A. oryzae* fermentation products are widely used as flavour enhancers in human foods. Similar effects of YC and AO on the acceptability of feeds to ruminants cannot be ruled out. The products certainly have a pleasant odour, and *Lyons* (1987) and *Rose* (1987) suggested that the ability of yeast to produce glutamic acid could benefit the taste of feedstuffs supplemented with YC. While palatability improvements can certainly do no harm, there is now strong evidence that fungal feed additives have a more fundamental metabolic effect.

ADULT RUMINANTS: INFLUENCE OF FUNGAL FEED ADDITIVES ON DIGESTION

An improved feed intake would be expected to occur if fibre digestion in the rumen were increased. The latter is seen sometimes, but not always, when the measurement made is of total tract digestibility. *Wiedmeier* et al. (1987) found increases in DM, ADF and hemicellulose total tract digestibility with AO, YC and combined AO and YC in dry cows fed a mixed forage/concentrate diet. In three trials with cows, *Gomez-Alarcon* et al. (1990) observed that, with the exception of a diet containing high forage (63% alfalfa hay), AO caused increases in total tract DM, ADF and NDF digestibility. Similar responses to AO were observed in grazing steers, but only in one of three summer months (*Caton* et al., 1993). AO increased DM digestibility in a trial in which no significant intake response was seen (*Gomez-Alarcon* et al., 1988). In contrast, *Arambel* and *Kent* (1988), *Arambel* et al. (1987, 1990), *Oellermann* et al. (1990) and *Sievert* and *Shaver* (1993) observed no changes in digestibility with heifers or cows fed AO, and *Judkins* and *Stobart* (1988) found no increase in digestibility in weathers fed AO. *Harrison* et al. (1988) and *Williams* and *Newbold* (1990) reported no effect with cows fed YC. A combined AO-YC product had no effect on the extent of digestion in heifers fed

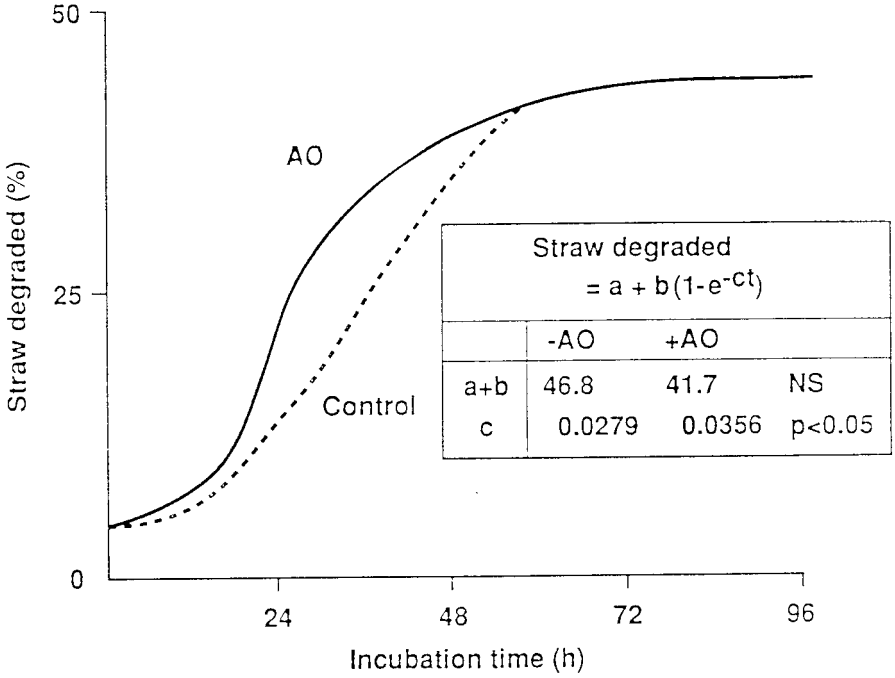

Figure 2: Influence of *A. oryzae* extract (AO) on the degradation of barley straw suspended in nylon bags in the rumen. The rate, c, but not the extent, a + b, of straw degradation was improved by AO. From: *Fondevila* et al. (1990).

a 50% orchardgrass hay diet (*Firkins* et al., 1990).

Total tract digestibility can, however, conceal profound changes in the site or rate of degradation of fibre in the tract. If fibre degradation in the rumen is stimulated, this might simply reduce the residue of material which is normally broken down in the hindgut, producing the same overall digestibility. *Gomez-Alarcon* (1988) found that AO and SC stimulated fibre breakdown in the rumen, effectively shifting more of the digestion to the rumen from the lower gut. *Fondevila* et al. (1990) observed that AO stimulated by 28% the rate of breakdown of straw suspended in nylon bags in the rumen, although the final extent of degradation was unchanged (Figure 2). Feeding YC to sheep gave similar effects on the digestion of hay in the study of *Chademana* and *Offer* (1990). No changes were found in OM, NDF or gross energy digestibility of three diets of differing forage-concentrate ratio, and the 48 h degradability of hay in the rumen was unchanged. However, 24 h OM degradation was increased by 11.6, 15.6 and 12.1% in low, medium and high forage diets respectively. Similar patterns of *in situ* breakdown were observed by *Campos* et al. (1990) with AO in non-lactating dairy cows fed mainly corn stover. Improved digestibility at 24 but not 48 h also occurred with AO *in vitro* (*Newbold* et al., 1991) and in the sheep rumen (*Newbold* et al., 1992a).

Another important factor that can affect intake is the outflow rate of digesta from the rumen. The results with fungal feed additives have been mixed, however. *Wiedmeier* et al. (1987) found decreased liquid and particle outflow rates

with AO and increases in the same rates with YC. Liquid outflow was stimulated by YC in growing steers under feedlot conditions (*Adams* et al., 1981), but was not affected significantly in sheep receiving YC (*Chademana* and *Offer*, 1990). Sheep receiving straw showed no change in liquid outflow (*Fondevila* et al., 1990).

It can be concluded therefore that the enhanced intake which drives production responses to fungal feed additives is most likely due to an improved rate of breakdown of foodstuffs in the rumen. The stimulation, predominantly of fibre digestion, need not affect the final ruminal degradability or total tract digestibility.

INFLUENCE OF FUNGAL FEED ADDITIVES ON RUMEN FERMENTATION

Ruminant nutrition studies are often accompanied by estimates of some easily measured parameters in rumen fluid, including pH, volatile fatty acids (VFA) and ammonia concentrations. These often help to explain the effects of different dietary manipulations on host animal nutrition. With fungal feed additives, however, the trends that can be discerned, with the possible exception of rumen pH, tell us little about how YC and AO work.

The effects of YC and AO on VFA and ammonia concentrations in rumen fluid were summarised previously (*Dawson*, 1990; *Martin* and *Nisbet*, 1992; *Wallace* and *Newbold*, 1992, 1993). The effects are always small and often insignificant, and it is our view that even where the differences reach statistical significance the biological significance is low. Possibly of much greater significance are findings that YC stimulated the rate of VFA production from different substrates *in vitro* in rumen fluid taken from sheep receiving YC (*Gray* and *Ryan*, 1989; *Ryan* and *Gray*, 1989). The significance of fermentation rate will be discussed below.

Methane production represents a substantial energy loss to the ruminant (*Hungate*, 1966). It is also intimately associated with the relative proportions of VFA that are produced (*Demeyer* and *van Nevel*, 1975) and the deamination of amino acids (*Russell* and *Martin*, 1984). In two studies *in vitro*, an increase in methane production was observed when YC was added to a batch system (*Martin* et al., 1989; *Martin* and *Nisbet*, 1990). Surprisingly, increased hydrogen production was also observed (*Martin* and *Nisbet*, 1990). A decreased proportion of methane in the headspace gas was found when AO was added to a semi-continuous rumen fermenter (Rusitec; *Frumholtz* et al., 1989), and methane production was decreased in calves when YC was included in the diet (*Williams*, 1988). Clearly more *in vivo* studies are required to establish how significant the effects of fungal feed additives are on methane production.

Rumen pH is one of the most critical determinants of rumen function, particularly for the cellulolytic bacteria, which fail to grow at pH 6.0 and below (*Stewart*, 1977). The fall in pH that results from increasing concentrate in the diet causes, in part at least, negative associative effects between forages and concentrates: the degradability of the fibrous components of the diet is inhibited by adding concentrate above a certain proportion (*Mould* et al., 1983). Fungal feed additives usually appear to increase rumen pH slightly (*Wiedmeier* et al., 1987; *Gomez-Alarcon* et al., 1990; *Oellermann* et al., 1990; *Fiems* et al., 1993), although this does not always happen (*Arambel* et al., 1990;

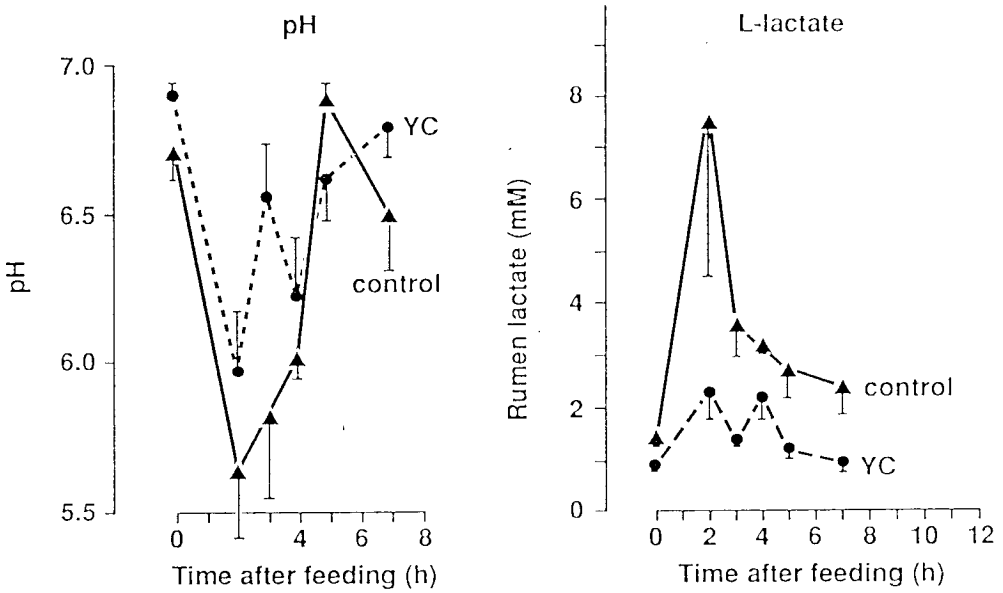

Figure 3: Influence of yeast culture on rumen pH and lactate concentrations following the addition of concentrate feed to the rumen. From: *Williams* et al. (1991).

Chademana and *Offer*, 1990; *Fondevila* et al., 1990; *Gomez-Alarcon* et al., 1990; *Quigley* et al., 1992; *Piva* et al., 1993; *Sievert* and *Shaver*, 1993) and in some experiments YC actually caused a fall in rumen pH (*Harrison* et al., 1988; *Edwards* et al., 1990). Increases in pH have also been recorded in *in vitro* fermentation systems (*Frumholtz* et al., 1989; *Dawson* et al., 1990).

Perhaps the most crucial aspect of how fungal feed additives affect rumen fermentation is often concealed within the experimentally derived VFA and ammonia concentrations and pH values that are reported. *Harrison* et al. (1988) noted that variation in ammonia concentrations in rumen fluid from cows receiving yeast culture was less than controls, and microbial numbers were similarly more stable. They concluded that ruminal fermentation was more stable in cows receiving yeast culture supplement. Experiments with steers fed barley with hay in the work of *Williams* (1989) and *Williams* et al. (1991) illustrate this point (Figure 3). Post-feeding peaks in lactate concentration and troughs in pH were markedly decreased in animals receiving YC.

Microbial yield is a vital factor in ruminant nutrition, because in most diets rumen microbial protein is the main source of amino acids available for absorption by the animal; much of the protein consumed by the animal is hydrolysed by proteolytic rumen bacteria. Effects of fungal feed additives on microbial yield are variable. *Wanderley* et al. (1987) found that AO had no effect on yield, whereas *Gomez-Alarcon* et al. (1990) observed that microbial yield was increased by AO in two trials out of three done with cows. In the third trial, where AO had no effect, YC was included as another treatment, and it also failed to stimulate the yield. *Wanderley* et al. (1987) found that protein flow was increased, however, and *Williams* et al. (1990) measured protein flow at the duodenum of sheep, which tended to be increased by YC, leading to in-

creased absorption of non-ammonia N. Urinary allantoin measurements in bulls implied that YC had improved microbial yield (*Edwards* et al., 1990). In the study of *Wanderley* et al. (1987), it appeared that the increased protein flow was a consequence of both increased microbial protein and increase undegraded dietary protein in the digesta at the duodenum.

EFFECTS OF MICROBIAL FEED ADDITIVES ON RUMEN MICROBIOLOGY

Yeasts and moulds occur naturally in the microbial community of the rumen (*Lund*, 1974, 1980). Up to 1.3 x 10^5 yeasts/ml grew when dilutions of bovine rumen fluid were incubated at 25°C, but only 3.5 x 10^3/ml grew at 39°C, suggesting that the yeasts present normally are essentially transient members of the community, entering with the fodder (*Lund*, 1974). Nine different species were identified, none of which was a *Saccharomyces* (*Lund*, 1974). Yeast numbers were similar in sheep (*Newbold* et al., 1990). A natural yeast population was undetectable in some roughage-fed steers, and yeasts were also undetectable when the rumen fluid was used in *in vitro* continuous cultures (*Dawson* et al., 1990). Thus yeasts, and particularly *S. cerevisiae*, are not normally significant members of the rumen microbial community. The temperature (39°C) and chemical composition of rumen fluid tended to be inhibitory to growth of *S. cerevisiae in vitro* (*Arambel* and *Tung*, 1987).

Numbers of aerobic fungi in the rumen of straw-fed sheep were 4.2 x 10^5/ml in control animals and 5.8 x 10^5/ml in those receiving AO (*Fondevila* et al., 1990). The aerobic fungal population was smaller in cows fed a mixed diet (1.7 x 10^3/ml), and no significant increases occurred in response to increasing amounts of AO (*Oellermann* et al., 1990).

Substantial increases in the total viable count of anaerobic bacteria (TVC) in the rumen when ruminants were fed fungal feed additives were first observed *in vivo* with AO (14%) and YC (30%) by *Wiedmeier* et al. (1987). *Harrison* et al. (1988) subsequently reported a 58% increase in TVC with YC, and *Dawson* et al. (1990) a nearly five-fold increase in TVC when steers were fed YC. Large increases have been also observed *in vitro*. *Frumholtz* et al. (1989) found a 79% increase in TVC with AO, and substantial increases were found in subsequent studies with AO (*Newbold* et al., 1991, 1992a,b), and an increase of more than ten-fold occurred in response to YC in the continuous culture of *Dawson* et al. (1990). *Wallace* and *Newbold* (1993) summarised available data for yeast; the average stimulation in all the studies excluding that of *Dawson* et al. (1991) was 52%.

Clearly such large increases in TVC do not reflect changes in the total bacterial protein present, in view of small or no effects on microbial yield. Several explanations are possible. Some, such as the possibility that the average cell size decreases with fungal feed additives, or that clumps of cells dissociate to form more colony-forming units, are improbable. It is more likely that more of the bacteria present are viable, i.e. fewer are dead, when fungal feed additives are used. Thus YC and AO must in some way improve conditions for the growth of rumen bacteria.

The growth of cellulolytic bacteria is also stimulated by fungal feed additives (*Wallace* and *Newbold*, 1993). Popula-

tion sizes *in vivo* tend to increase proportionally by a little more than the increase in total population (*Wiedmeier* et al., 1987; *Harrison* et al., 1988; *Arambel* et al., 1990; *Dawson* et al., 1990; *Newbold* et al., 1992b), but this does not always happen (*Fondevila* et al., 1990; *Newbold* et al., 1992a). *In vitro* the effect on cellulolytic bacteria is sometimes considerably greater than on the total population (*Frumholtz* et al., 1989; *Arambel* et al., 1990; *Dawson* et al., 1990), although again this does not always hold (*Newbold* et al., 1991).

Ciliate protozoa comprise up to half of the total microbial biomass in the rumen (*Williams* and *Coleman*, 1988) and they are primarily responsible for the wasteful breakdown and resynthesis of bacterial protein that reduces microbial yield (*Demeyer* and *van Nevel*, 1979; *Wallace* and *McPherson*, 1987). They also contribute to cellulolysis (*Coleman*, 1985; *Williams* and *Coleman*, 1988). Yet, despite their evident importance, few protozoal counts appear to have been reported in ruminants fed fungal feed additives. Protozoal numbers were reduced by nearly half in Rusitec when AO was added (*Frumholtz* et al., 1989; *Newbold* et al., 1993), but numbers were unaffected in sheep (*Newbold* et al., 1992a,b) and tended to increase with AO in cows (*Oellermann* et al., 1990). AO had no effect on the predatory activity of protozoa on bacteria *in vitro* (*Newbold* et al., 1992b).

The third major category of rumen microorganisms, namely the anaerobic fungi, which are highly cellulolytic (*Orpin* and *Joblin*, 1988), have likewise received little attention with regard to fungal feed additives. AO tended to increase fungal numbers in the rumen digesta of cattle receiving AO (*Oellermann* et al., 1990). A second fungus, not identified, was present with *Aspergillus* attached to fibre particles in the duodenum of cattle receiving AO (*Wanderley* et al., 1985). Anaerobic fungi were less numerous than aerobic fungi in the bovine rumen and were not increased by AO (*Oellermann* et al., 1990). When AO was added directly to pure cultures of *Neocallimastix frontalis, Neocallimastix patriciarum* and *Sphaeromonas communis*, it had no influence on gas production by these major species of rumen anaerobic fungi (*Newbold* et al., 1992b). Thus most of the available evidence suggests that fungal feed additives have little effect on the natural population of anaerobic fungi growing in the rumen.

OTHER EFFECTS OF FUNGAL FEED ADDITIVES

A number of other effects have been attributed to fungal feed additives that may or may not be directly associated with their mode of action. In cattle subjected to high ambient temperatures, AO reduced rectal temperature and heat stress (*Huber* et al., 1986; *Huber*, 1987; *Higginbotham* et al., 1993). YC may also affect mineral metabolism, presumably due to the ion-binding properties of its cell wall (*Rose*, 1987). Improved zinc nutrition may explain some of the effects of YC (*Williams*, 1988). One of the most surprising, and potentially most significant findings with YC has been that the amino acid profile of protein reaching the duodenum was significantly altered by YC (*Erasmus* et al., 1992). The increased flow of methionine would be expected to be of importance because methionine is often the first-limiting amino acid in ruminant nutrition (*Wallace*, 1994). At present none of the proposed modes of action of YC can account for this observation.

POST-RUMINAL EFFECTS

The findings that viable yeast survived passage through the tract to increase numbers in the duodenum, ileum and faeces of sheep (*Newbold* et al., 1990; *Fiems* et al., 1993) and *Aspergillus* spores were present in duodenal digesta of cows receiving AO (*Wanderley* et al., 1985) could have important implications for a second site of action of fungal feed additives. Possible post-ruminal effects of fungal feed additives have been largely ignored until now. It is possible that the benefits noted with horses receiving YC, such as improved nutrient digestibility, which arise from stimulation of the caecal microflora (*Pagan*, 1990) could also occur with ruminants.

POSSIBLE MODES OF ACTION

It is appropriate at this point to separate discussion about YC and AO, since it is becoming clear that the two types of additive have different modes of action on the rumen microbial population.

Yeast culture

Dawson (1987) obtained data from *in vitro* experiments which implied that *S. cerevisiae* might grow in the rumen. Subsequent experiments suggest that substantial growth of yeast is unlikely to occur, however. Yeast numbers increased from 2.5×10^5 to 4.7×10^5/ml 4 h after feeding in cows receiving YC (*Harrison* et al., 1988), and when YC was fed to sheep, yeast numbers in rumen fluid increased from 1.5×10^3 to 3.3×10^5/ml after 1 h (*Newbold* et al., 1990). When numbers in the sheep rumen were extrapolated back to zero time, the population of yeast corresponded to the number of viable cells added as YC with the feed. Numbers then declined at a rate of 0.17 h^{-1} , i.e. somewhat faster than would be expected simply from liquid dilution, indicating that any net growth of yeast in the rumen was insignificant and that cell death in fact occurred. Similar conclusions were made by *Arambel* and *Tung* (1987) using vivar chambers fitted with different sizes of membrane filters. *In vitro* continuous cultures (*Dawson* et al., 1990) confirmed that viable yeasts do not increase in number in rumen fluid.

The inability of yeast to grow in the rumen should not be confused with a lack of metabolic activity, however. *Ingledew* and *Jones* (1982) found that *S. cerevisiae* was metabolically active in rumen fluid for up to 6 h. Yeast extract that did not contain live cells did not stimulate the growth of *F. succinogenes* on cellulose *in vitro* in the same way as *S. cerevisiae* (*Dawson*, 1990). Similarly, autoclaved YC (*Dawson* et al., 1990) was ineffective in stimulating bacterial numbers in mixed fermentations *in vitro*. YC that had been sterilised by gamma-irradiation rather than autoclaving retained most of its stimulatory activity (*El Hassan* et al., 1993). Either a heat-sensitive nutrient must be destroyed by autoclaving, or YC has a metabolic activity that is destroyed by heat but not irradiation.

Rose (1987), *Dawson* (1987) and many others since have suggested how the fungal feed additives work in terms of their many different individual effects on the animal and on rumen fermentation. It seems unlikely, however, that there are more than one or two critical primary sites at which YC or AO act to exert their ultimate nutritional benefit. Fungal feed additives must interact at

the cellular or molecular level in the animal, probably in the rumen, to cause these primary effects, which then have secondary consequences and so on until the productivity benefit occurs. It is therefore vital to distinguish the important primary effects from the secondary ones if an understanding of fungal feed additives is to be obtained.

The imaginative work of *Williams* (1989) and *Williams* et al. (1991) suggested that stabilisation of rumen pH was the reason for the improved microbial growth. However, despite earlier speculation about the properties of yeast cell walls (*Rose*, 1987), YC has no influence whatever on the buffering capacity of rumen fluid (*Ryan*, 1990; *Wallace* and *Newbold*, 1992). In any case, a buffering capability would not be expected to be heat-labile. The stabilisation of rumen pH must therefore have been a secondary effect of YC rather than the primary effect.

Lactic acid has a lower pK_a than the VFA, so the lower lactate concentrations observed in *Williams* (1989) and *Williams* et al. (1991) experiments and others (*Newbold* et al., 1990) could conceivably have been responsible for the increased rumen pH. The strain of yeast used in Yea-sacc does not assimilate lactate, although other strains of *S. cerevisiae* do (NCYC, 1990), therefore the yeast did not ferment the lactate in these experiments. Alternatively, substances in YC might have stimulated the removal of lactate by indigenous rumen bacteria, as shown in pure culture with the lactate-fermenting rumen bacterium, *Selenomonas ruminantium* (*Nisbet* and *Martin*, 1991). The dicarboxylic acids fumarate and malate stimulated lactate transport, as did malate-containing soluble extracts of YC (*Nisbet* and *Martin*, 1991). The malate content of fungal feed additives was not high, however, considering the small amounts of product that are effective in the animal. Furthermore, autoclaving would not be expected to destroy malate, yet the products' activity was destroyed by autoclaving. It is possible that yeasts and fungi actually produce dicarboxylic acids in the rumen and thereby stimulate lactate uptake by rumen bacteria. Finally, malic acid infused into the rumen via the rumen cannula did not evoke the same response as YC (*Wallace* et al., 1993).

Another possibility is that decreased lactate production could have been caused by a slower fermentation of dietary sugars - lower concentrations of soluble sugars were present in steers receiving YC (*Williams* et al., 1991). Rapid bacterial growth in the rumen tends to cause increased lactate production (*Hungate*, 1966), and removal of sugars may have slowed growth sufficiently to decrease lactate production. Confirmation of this theory has not appeared. The main problems with the lactate-pH link are that the small quantities of lactate (up to 8 mM), observed in *Williams* (1989) and *Williams* et al. (1991) experiments would not give a significantly different pH in rumen fluid to equivalent amounts of VFA (*Wallace* and *Newbold*, 1992), and that the pH increases that are observed in most experiments are very small or insignificant. Furthermore, the stimulation of the rumen cellulolytic bacterium, *Fibrobacter succinogenes*, by yeast *in vitro* (*Dawson*, 1990) almost certainly would not be explained by a change in culture pH, as only 10^4 yeast cells/ml were added. It can be concluded, therefore, that decreased rumen lactate concentrations must be a secondary consequence of fungal feed additives arising from, but not responsible for, improved bacterial growth.

Having eliminated some possible explanations, other suggestions remain to be evaluated in terms of the primary mechanism by which bacterial TVC is

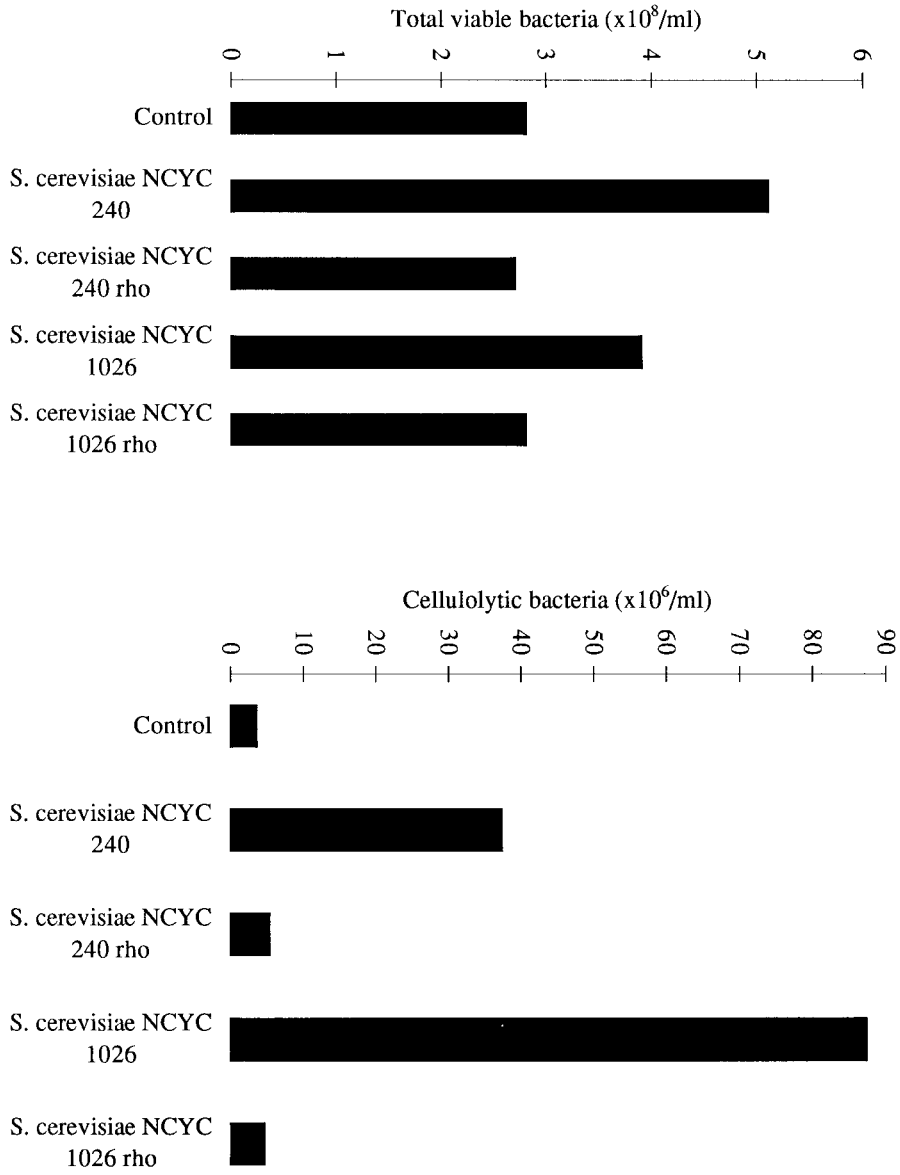

Figure 4: Influence of respiration-deficient (rho) mutants of yeasts and their parent strains on the stimulation of total viable and cellulolytic bacteria in the rumen simulation technique. From: *Newbold* et al. (1994b).

increased. It has been suggested that YC may remove toxic factors in rumen fluid that inhibit the growth of rumen bacteria. The removal of toxic metal ions is possible for yeast, with its highly ionic cell wall (*Rose*, 1987), but again a loss of stimulation by autoclaving would not be consistent with this mode of action. Scavenging of molecular oxygen (*Rose*, 1987) is another possibility. Molecular oxygen is much more toxic to *F. succinogenes* and other rumen bacteria than increased Eh (*Marounek* and *Wallace*, 1984), so

traces of O_2 could be detrimental even without changing Eh. Recent work with different strains of yeast and respiration-deficient yeast mutants demonstrates that the ability of yeast to stimulate the viable count in the rumen depends on its respiratory activity (Figure 4; *Newbold* et al., 1993, 1994a,b). It is proposed that yeast removes some of the O_2 that occurs in ruminal fluid at various times during the daily feed cycle (*Hillman* et al., 1985) and, therefore, prevents toxicity to the ruminal anaerobes. Thus the effectiveness of YC will depend on the degree to which the diet and animal induce the entry of oxygen into or removal of oxygen from the rumen and therefore may explain the variation in responses obtained.

Aspergillus oryzae extract

The precise mode of action of AO has received less attention than YC. Some of the same observations apply. Substantial growth of *A. oryzae* does not occur in the rumen, and autoclaving destroys the stimulation, while irradiation is less detrimental (*Newbold* et al., 1991). The dicarboxylic acids present in the extract stimulate lactate production by *Selenomonas ruminantium* (*Nisbet* and *Martin*, 1990) and *Megasphaera elsdenii* (*Waldrip* and *Martin*, 1993), but once more the quantity of dicarboxylic acids present does not seem to be sufficient to have a major effect on lactate metabolism by the mixed rumen population (*Varel* et al., 1993).

It is much more likely that it is the enzymes present in the extract that are responsible for the activity of AO in the rumen. AO contains enzymes capable of the digestion of plant cell wall material. These are believed to include cellulase, xylanase, and phenolic acid esterases (*Varel* et al., 1993). Furthermore, AO affected the digestion of different types of plant fibre *in situ* and *in vitro* in different ways, which may account in part for the variation in production and fermentation responses described above. *Gomez-Alarcon* et al. (1990) reported that AO stimulated digestion of alfalfa hay but not sorghum grain or wheat straw. *Beharka* and *Nagaraja* (1993) found a stimulation of digestion of alfalfa hay as well, and of bromegrass hay but not pure cellulose, endophyte fescue, wheat straw, corn silage or prairie hay. Bromegrass and switchgrass breakdown were found to be accelerated *in vitro* by *Varel* et al. (1993). Identification of the precise enzyme activity responsible for these stimulations would be an important step forward in understanding and manipulating rumen fermentation. Different species in the genus *Aspergillus* may give effects comparable to *A. oryzae*. *Aspergillus niger* was at least as effective as *A. oryzae* in enhancing nutrient digestion in cows (*Campos* et al., 1990). *In vitro* digestibility trials suggested that species of *Trichoderma* and *Penicillium* would be much less active than the two *Aspergillus* species (*Tapia* and *Herrera-Saldana*, 1989).

BACTERIAL PROBIOTICS FOR ADULT RUMINANTS

Relatively few experiments have been done in adult ruminants with the types of bacterial preparation that are used in young ruminants or monogastric animals. *Jaquette* et al. (1988) and *Ware* et al. (1988) reported significant increases (6.2 and 5.7% respectively) in milk production from cows receiving *L. acidophilus*. The mode of action of a lactobacillus preparation in the rumen is difficult to imagine. Lactobacilli produce lactate, sometimes to the severe

detriment of the animal in cases of lactic acidosis (*Slyter*, 1976). Seven species of rumen bacteria were unaffected by lactobacilli or enterococci that are used in bacterial probiotics to inhibit pathogens (*Newman* et al., 1990). It is nonetheless possible that less common, possibly detrimental species are inhibited by lactobacilli. Bacterial probiotics may have no advantage over fungal products in the adult animal, however. A mixed YC + *Lactobacillus* + *Streptococcus* preparation was little different to YC alone in its influence on rumen fermentation (*Dawson* et al., 1990).

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LACTIC ACID BACTERIA AND HUMAN HEALTH

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INTRODUCTION

This review will focus on the general considerations of lactic acid bacteria and what is known of their direct effect on human health. Much research, necessarily, has focused on *in vitro* or animal system development. These studies are an important first step in developing a sense of potential outcomes and mechanisms. However, these studies can always be criticised due to their questionable significance to human physiology. Therefore, this review will focus on studies which have been done in humans. These studies take two principle forms: epidemiological studies, which are rare, and direct clinical studies, which are diverse.

PROBIOTICS

A probiotic has been defined by *Havenaar* and *Huis in 't Veld* (1992) as "a mono- or mixed-culture of live microorganisms which, when applied to animal or man, affect the host beneficially by improving the properties of the indigenous microflora". This definition somewhat limits the mechanism of probiotics, since, for example the use of cultures to deliver lactase to aid with lactose digestion would not fit under this definition. In addition, improvement of the properties of indigenous microflora may be more difficult to substantiate than the more empirical benefit of the host. For this reason, simplifying the definition to "a culture of live microorganisms which, when applied to animal or man, affect the host beneficially" may be more suitable for pragmatic purposes.

Bacteria proposed for probiotic uses are usually categorised as lactic acid bacteria. This includes a broad group of Gram-positive, non-sporeforming, catalase-negative rods and cocci that ferment carbohydrates to form lactic acid as their primary metabolic end-product. The primary genera associated with this general grouping are *Lactococcus, Lactobacillus, Pediococcus, Leuconostoc* and *Streptococcus* (usually limited to the *S. thermophilus* species). In discussions on lactic acid bacteria used for probiotic purposes, the genus *Bifidobacterium* is frequently included.

MECHANISMS OF INFLUENCE

Many have advanced the theory that certain lactic acid bacteria, primarily those which normally reside in the human intestine, may exert a positive influence on human health (for reviews, see *Fernandes* et al., 1992; *Hitchins* and *McDonough*, 1989; *Gurr*, 1992; *Fuller*, 1991; *Gorbach*, 1990). The human in-

Table 1: Hypothesis: intestinal functioning of probiotic cultures

Compounds possibly present in the intestine	Action of Intestinal Bacteria	Influence on Health
	Potential negative influence	
bile salts*	→	3oxo-chol-4en-24oic acid
hormones	→	estradiol
peptides*	→	phenolic compounds, amines
steroids	→	polycyclic aromatic hydrocarbons
		Potential beneficial influence
cholesterol	→	cholestenone
lactose	→	glucose and galactose
carbohydrates	→	organic acids

testinal tract is continuously challenged by a variety of bacteria and viruses, some of which are capable of pathogenic activity or producing toxic metabolites. Lactic acid bacteria are not enteropathogenic or enterotoxigenic, and are known for their ability to suppress the growth of species of enteric bacteria *in vitro*. Although physiological conditions *in vivo* are vastly different than these *in vitro* model systems, microbial behaviour in these *in vitro* model systems is still used as evidence of their potential to modulate the intestinal microbial ecosystem. The presence of lactic acid bacteria in high numbers in the intestine is postulated to limit the growth, metabolism or survival of other enteric bacteria, which may in turn limit pathogenic or toxigenic effects of these bacteria. Negative effects of intestinal bacteria have been suggested (Table 1), and include production of toxic metabolites and carcinogens.

Ultimately, the human intestinal system is notorious for its complexity, and the human being reluctant a reluctant subject for certain types of direct experimentation. For these reasons, the mechanisms of probiotic action in humans remains largely unproved.

MEASUREMENT OF PROBIOTIC EFFECTS

Investigation into the effects of probiotics on human health has expanded across a broad range of clinical and *in vitro* systems. *In vitro* systems, including tissue cell culture analysis of adherence potential, identification from probiotic strains of bacteriocins which may play a role in competitive exclusion in the intestine, metabolic studies to identify antagonistic substances produced by probiotic strains, colonisation studies in experimental animals, and other systems have all contributed to a basic understanding of the potential of these microbes. However, the real test of effectiveness is determined by how these *in vitro* activities may affect clinical conditions.

Assays of the activity of probiotics directly in humans has also been varied. In some instances, these assays are not optimally conducted, since insufficient attention has been paid to microbiological factors which may affect probiotic

Table 2: Carcinogens or suspected carcinogens naturally present in food (adapted from *Scheuplein*, 1992)

Food	Carcinogen
garlic	alkyl isothiocyanate
chilies	capsaicin
oranges	d-limonene
mushrooms	hydrazines
cereals, nuts contaminated with mold	aflatoxin
herbal teas	pyrrolizidine alkaloids
spinach, beets, lettuce, radishes	nitrates → nitrosamines
fish and shellfish	polyaromatic hydrocarbons

performance. Studies using human subjects fed probiotics or probiotic-containing foods have measured changes in faecal microbial populations, faecal microbial enzyme activity, intestinal chemistry, survival through the gastrointestinal tract, colonisation (biopsy methods), clinical conditions (gastrointestinal and vaginal infections, intestinal symptoms), breath hydrogen (measure of lactose digestion), and blood chemistry (including blood lipids and immunological factors). Taken together with the *in vitro* research, these studies have begun to paint a picture of the way probiotic cultures may affect human health.

EPIDEMIOLOGICAL EVIDENCE

Diet and cancer

Epidemiologists estimate that from 10 - 70% of all cancer deaths can be attributed to diet, and that the risk stems predominately from the food, not from additives, pesticides, or contaminants (*Scheuplein*, 1992). However, a recent publication from the Council on Scientific Affairs (1993) cautions that a simple solution does not exist to conclusively link specific nutritional factors in cancer progenesis. However, carcinogenic substances in food (Table 2) are suspect for contributing to cancer incidence.

Drasar and *Hill* (1972) advanced the theory that the "internal environment" of the intestinal tract may affect cancer rates, especially colon, breast and stomach. Transformations of foodstuffs, endogenously-produced compounds, or microbial by-products by the plethora of microbes present in the intestinal tract into harmful substances may lead to the progenesis of different types of cancer. Unfortunately, although indirect experimentation on animals or using *in vitro* systems suggests that probiotic cultures can have a positive effect on cancer incidence, epidemiological evidence supporting these claims in humans is sparse. Since direct experimentation on humans and cancer is not possible, these epidemiological studies are critical to gaining an understanding of potential effects.

Breast cancer

One epidemiological study was conducted on breast cancer incidence and fermented milk, Gouda cheese and milk consumption in the Netherlands (*van 't Veer* et al., 1989). This study involved 133 breast cancer patients and 289 con-

trols. Breast cancer subjects were asked to recall dietary consumption patterns for the 12 months prior to diagnosis. The study found that the consumption of fermented milk products was significantly higher among the controls than the breast cancer patients. After correction for dietary fat intake, Gouda cheese consumption also correlated with lower incidence of breast cancer. Milk consumption was not significantly associated with breast cancer risk. The authors speculated that the protective effect of fermented milk products may be due to the interference of lactic acid bacteria with entero-hepatic circulation or their stimulation of the immune system. However, they caution that further observational and experimental research are needed to substantiate any effect. The primary weakness of this study lies with the nature of data collection. Recall studies are inherently less reliable than direct measurement. Furthermore, dietary patterns during 12 months prior to diagnosis are largely insignificant to the progenesis of a disease which may be decades in development. This study is defended by the observation that consumption of fermented milk products in the Netherlands is thought to be a life long pattern. Firm establishment of this fact is essential for proper interpretation of these results.

HUMAN CLINICAL STUDIES

Diarrhoeal illnesses

The assessment of the effect of lactic cultures on diarrhoeal illnesses is a very general topic. Diarrhoea is a single symptom, but with a myriad of progenitors. Disruption of the chemistry, physiology or microbiology of the intestinal system can result in an influx of fluids into the bowel, resulting in clinical diarrhoea. Bacteria, protozoa, viruses, as well as chemical substances (e.g., antibiotics, oligosaccharides, non-digestible fibres) can influence the microbial or chemical balance in the intestine and lead to diarrhoeal illness.

Many human clinical studies have been published on the effect of probiotics or probiotic-containing products on diarrhoeal illnesses. These studies are summarised in Table 2, and some of the positive studies will be discussed below.

The onset of diarrhoeal illness during or after the course of antibiotic therapy is a common side effect, in some cases leading to pseudomembranous colitis. Virtually all cases of pseudomembranous colitis resulting from antibiotic therapy can be traced to a toxin produced by *Clostridium difficile* (*George*, 1980). This is a disease of the large bowel, and is unique in that the causative agent, *C. difficile*, is commonly present in the normal bowel, but is not pathogenic until the intestinal microecology is disturbed by antibiotic therapy. Therefore it appears that the normal intestinal flora suppresses growth and toxin formation by *C. difficile*. In most cases, *C. difficile* colitis is cured with focused antibiotic therapy. However, in a small number of cases, the disease relapses, and is refractory to antibiotic treatment. The effect of probiotic therapy on relapsing *C. difficile* pseudomembranous colitis was tested in a limited study by *Gorbach* et al. (1987). Five patients with relapsing *C. difficile* colitis consumed 10^{10} cfu/day dried *Lactobacillus casei* GG suspended in milk for 7-10 days. After treatment, no additional relapses occurred over a follow-up period of 4 months to 4 years.

Additional experiments with the GG strain have been done on diarrhoea

Table 3: Summary of studies to determine the effect of lactic cultures on diarrheal illnesses (ETEC, enterotoxigenic *E. coli*)

Clinical target	Probiotic culture used	Total number of subjects	Effect on diarrhea in treatment	Culture format/ daily culture dose groups	Reference	Placebo controlled?
rotavirus diarrhea 82%	*L. casei* GG	71 children	_ duration by 1 day	yogurt or dried powder 10^{10}-10^{11} cfu/day	Isolauri et al., 1991	yes
rotavirus diarrhea	*L. casei* GG	22 children	_ duration by 1.4 days	10^{10}-10^{11} cfu/day	Kaila et al., 1992	yes
relapsing *C. difficile* colitis	*L. casei* GG	5 adults	follow-up with no relapses: 4 mo - 4 yr	concentrate in 5 ml milk 10^{10} cfu/day	Gorbach et al., 1987	no
erythromycin-associated diarrhea	*L. casei* GG	16 healthy adults	_ incidence	yogurt $2x10^{10}$ cfu/day (est.)	Siitonen et al., 1990	yes
travellers' diarrhea	*L. casei* GG	756 travellers	_ incidence in one of two groups studied	powder $2x10^{9}$ cfu/day	Oksanen et al., 1990	yes
intractable diarrhea	bifidobacteria and/or *L. casei* dried or yogurt	15 children	clinical improvement within 3 to 7 days	10^{10}	Hotta et al., 1987	no
deliberate ETEC infection; neomycin-associated diarrhea	Lactinex®	40 young adults	no effect on ETEC diarrhea; variable effect on neomycin-associated diarrhea	powder $7x10^{7}$	Clements et al., 1983	yes
deliberate ETEC infection	Lactinex®	48 young adults	no effect on ETEC diarrhea	powder $7x10^{8}$	Clements et al., 1981	yes
travellers' diarrhea	Lactinex®	50 healthy adults	no effect	powder $3\text{-}7x10^{9}$	Pozo-Olano et al., 1987	yes
acute-onset diarrhea	dried *L. acidophilus*, *L. bulgaricus* and *S. thermophilus*	94 children	no effect	powder $3\text{-}8x10^{8}$	Pearce and Hamilton, 1974	

Table 4: Comparison of daily dose and overall results from studies on effect of lactic cultures on diarrhoea illnesses

Daily Dose	Probiotic Used	Overall Effect	Reference
7×10^7	Lactinex®	-	Clements, et al., 1983
7×10^8	Lactinex®	-	Clements, et al., 1981
	Sweet Acidophilus Milk	-	Newcomer, et al., 1983
$3\text{-}7 \times 10^9$	Lactinex®	-	Pozo-Olano, et al., 1987
$3\text{-}8 \times 10^8$	Dried *L. acidophilus*, *L. bulgaricus* and *S. thermophilus*	-	Pearce and Hamilton, 1974
10^{10}-10^{11}	*L. casei* GG	+	Isolauri, et al., 1991
10^{10}	*L. casei* GG	+*	Gorbach, et al., 1987
10^{10}-10^{11}	*L. casei* GG	+	Kaila, et al., 1992
2.5×10^{10} (est.)	*L. casei* GG	+	Siitonen, et al., 1990
10^{10}	Bifidobacteria and/or *L. casei* , dried or yogurt	+*	Hotta, et al., 1987
2×10^9	*L. acidophilus* yogurt	+*	Salminen, et al., 1988

*not placebo controlled

caused by rotavirus, *C. difficile*, and erythromycin, as well as the less clearly defined "travellers' diarrhoea". Limitations, especially with the *C. difficile* study, where no controls are conducted and only five subjects are tested, can be readily identified. But overall, these studies suggest that *Lactobacillus* GG can limit the course of some diarrhoeal illnesses. Credit should be given to this focused research program for attempting to build a critical mass of data which can support clinical effects for a single strain. These researchers have, in general, paid attention to issues which are absent in many studies: quantitation of daily dose of a defined strain, maintenance of high daily doses, definition of a clinical system to test, and statistical analysis of results.

When reviewing the literature on anti-diarrhoeal effects, the importance of high daily dose to positive activity becomes apparent. Clinical studies which tested the effect of lactic cultures or fermented dairy products on incidence or duration of different diarrhoeal illnesses were grouped according to extent of effect and daily dose (Table 3). Unfortunately, not all clinical studies could be included in this analysis, since in some, daily dose is not provided or results cannot be determined to be positive or negative, since statistical analysis of the data was not conducted. Considering these limitations, Table 4 suggests that in general, studies which showed a positive effect, used high levels of culture. No studies using daily doses of $<10^9$ showed positive results.

Vaginitis

The lactobacilli are normal inhabitants of the human vaginal tract. Because of this, it is hypothesised that they may be able to exert an effect in the

overall microbial balance of the vagina, discouraging the presence or proliferation of pathogens. Some clinical and microbiological evidence supports this. One line of research has correlated the presence of H_2O_2-producing (but not non-producing) lactobacilli with decreased vaginal infections. *Eschenbach* et al. (1989) demonstrated that 6% and 96%, respectively, of women with or without bacterial vaginosis harboured H_2O_2-producing lactobacilli. 36% of women with bacterial vaginosis harboured H_2O_2-producing lactobacilli. These observations suggest that the antimicrobial effect of the H_2O_2, along with other factors such as vaginal pH, mediates pathogen suppression by vaginal lactobacilli. *Klebanoff* et al. (1991) demonstrated *in vitro* the toxic effects of producing lactobacilli *in vitro* against *Gardnerella vaginalis*. The H_2O_2-producing and non-producing lactobacilli used in the study were unfortunately not isogenic, but catalase-treatment eliminated the positive effect, supporting the role of H_2O_2.

In another line of research, the effect of yoghurt consumption on *Candida* infections in women was studied by *Hilton* et al. (1992). This study was a non-blinded study of crossover design. Nineteen women started the study and served as their own controls. Participants in the test arm of the study were asked to consume 8 oz of yoghurt per day for six months. In the control arm of the study, no yoghurt was consumed. Eight women, however, who began the study in the yoghurt arm, refused to enter the control arm of the study, due to clinical improvement. Of the remaining 11 subjects, results showed that the mean number of infections per six months was 1.66 in the control, and 0.38 in the yoghurt phase of the study, results which were highly statistically significant. Interestingly, these results were obtained with consumption, not direct application, of the lactic culture. This study needs to be repeated as a blinded, placebo-controlled study, but is suggestive of positive influence on yoghurt and the control of vaginal infections. Placebos composed of yoghurt with heat-killed bacteria would substantiate any role viable bacteria have in this clinical effect.

Faecal Metabolites

Repeated experiments have been conducted on enzymatic activity in faeces. Table 5 summarises these results. Certain enzymes produced by microbes present in human faeces are thought to play a role in the production of carcinogens, and therefore, overall cancer rates. Enzymes which have been assayed include ß-glucuronidase, nitroreductase, glycocholic acid hydrolase, urease, ß-glucosidase and steroid 7-α-dehydroxylase. The most consistent results of these studies indicate that ß-glucuronidase activity can be influenced by high daily doses of *L. acidophilus* or bifidobacteria strains. The results have been achieved with several different *L. acidophilus* strains. Choice of enzymes for these studies has been criticised by *Marteau* and *Rambaud* (1993). However, experiments with rats (*Goldin* and *Gorbach*, 1984) showed that *L. acidophilus* supplements reduce DMH-induced colon tumours. Although the mechanism of effect may not be proven as yet, the results suggest an overall positive effect. These results suggest that probiotic bacteria in adequate numbers can influence intestinal activity which may stimulate tumour formation.

Although information has been generated which suggests that *L. acidophilus* feeding can influence some metabolites of the faecal microflora, a recent study suggests that not all probiotic dietary intervention will necessarily have the same result. *Bartram* et al. (1994) studied changes in the faecal mi-

Table 5: Summary of the effects of oral consumption of lactic cultures on faecal enzyme activity in humans

Reference	Bacteria and daily dose	Reduction of faecal enzyme activity
Goldin, et al., 1992	*L. casei* GG frozen concentrate-10^{10} (8 subjects)	+ ß-glucuronidase
Ling, et al., 1994	*L. casei* GG (3 x 1010/day, in yogurt or yogurt + fiber) (64 female subjects)	+ ß-glucuronidase + nitroreductase + glycocholic acid hydrolase - urease - ß-glucosidase
Marteau, et al., 1990	galactooligosaccharides 2.5 g/day (which increase bifidobacteria)	+ ß-glucuronidase - nitroreductase
Goldin and Gorbach, 1984a	milk fermented with *L. acidophilus* (10^9), *B. bifidum* (10^{10}), and mesophilic cultures (10^{10}) (9 subjects)	+ nitroreductase - azoreductase - ß-glucuronidase + ß-glucosidase
Goldin and Gorbach, 1984b	*L. acidophilus* NCFM (10^{10}) (7 subjects)	+ nitroreductase - azoreductase + ß-glucuronidase
Ayebo, Angelo, and Shahani, 1980	*L. acidophilus* NCFM (10^9) *L. acidophilus* N-2 (10^9) (22 subjects)	+ nitroreductase + azoreductase + ß-glucuronidase
Goldin, et al., 1980	*L. acidophilus* DDS1 in milk ($1.4x10^9$) (12 subjects)	? ß-glucuronidase ? ß-glucosidase (no statistical analysis)
Ito, et al. (1993)	*L. acidophilus* ($4x10^{10}$) (7 subjects)	+ nitroreductase - azoreductase + ß-glucuronidase - steroid 7-α-dehydroxylase

+ : statistically significant positive results
- : negative results
? : results not definitive

croflora and chemistry in 12 healthy volunteers fed yoghurt made with normal yoghurt cultures (*S. thermophilus* and *L. bulgaricus*), serving as a control, or that same yoghurt supplemented with *Bifidobacterium longum* (daily dose > 5 x 10^8) and lactulose (daily dose 2.5 g). Faecal levels of *Bifidobacterium* increased when yoghurt with or without *Bifidobacterium* and lactulose was fed. However, faecal analysis revealed that no statistically significant differences in oro-anal transit time, wet or dry weight of stools, total, aerobic or anaerobic bacteria, concentrations of acetate, propionate, n-butyrate, isobutyrate, i-valerate, neutral sterols, or faecal pH could be attributed to bifidobacteria/lactulose consumption. The low level of daily dose of bifidobacteria may be

partially responsible for the lack of significant effects seen. Similar experiments with lactobacilli have fed in excess of 10^{10} lactobacilli per day. Significant differences were seen in the breath hydrogen levels in subjects consuming the yoghurt with bifidobacteria and lactulose, likely due to the faecal flora fermentation of the lactulose, and in the mouth-to-caecum transit time. The authors conclude that human faecal flora are refractory to probiotic and lactulose dietary intervention.

In interpreting these results in general, it should be remembered that the relationship between a statistically significant drop in microbial enzyme activity and improved health or decreased risk of cancer in humans, has not been determined. Until epidemiological studies confirm these results, the ultimate effect on human health must be considered speculative.

Toxic Metabolites

The effect of *Lactobacillus acidophilus* on the health of chronic kidney failure patients is currently being studied. These patients demonstrate bacterial overgrowth of the normally sparsely-populated small bowel. This bacterial growth results in the production of toxic metabolites, including dimethylamine (DMA), which when nitrosated in the gastrointestinal tract, forms the potent carcinogen, nitrosodimethyamine (NDMA). *Simenhoff* et al. (1994) have tested the effect of feeding 2×10^{10} freeze-dried, enteric-coated viable cells of *L. acidophilus* per day on levels of DMA in the blood of six chronic kidney failure patients. The study was placebo-controlled. Statistically significant ($p<0.01$) drops in the levels of blood DMA were seen for all patients during *L. acidophilus* feeding. The levels of DMA rebounded during placebo-feeding. Furthermore, NDMA levels decreased during *L. acidophilus* feeding. These effects were correlated with improved appetite and nutritional status of all patients. These results indicate that in these patients, feeding of *L. acidophilus* significantly altered the course of small bowel bacterial overgrowth, lending credence to the hypothesis that probiotic cultures can influence the microbiology and biochemistry of the gastrointestinal tract. These studies need to be expanded to include more, and perhaps less critically-ill, patients. If this type of result could be extended to less extreme cases of small bowel bacterial overgrowth, which can occur in people with atrophic gastritis, high blood pressure, diabetes, or heart disease, then the results could suggest a large impact on controlling toxic metabolites formed in the gastrointestinal system.

Lactose intolerance

It has long been believed that yoghurt is more readily digested than milk by lactose-intolerant people. There is now a significant body of scientific evidence which substantiates this effect (for reviews, see *Shah*, 1993; *Schaafsma*, 1993; *Savaiano* and *Levitt*, 1987; *Savaiano* and *Kotz*, 1988). It has been found that yoghurt containing viable yoghurt cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) consumed by lactose intolerant subjects resulted in lower breath hydrogen production and fewer symptoms than consumption of milk. It is thought that these lactase-containing yoghurt cultures deliver lactase to the intestine mediating the *in vivo* digestion of lactose to glucose and galactose before intestinal bacteria can degrade the lactose into more troublesome byproducts. Also contributing to better digestion of lactose in yoghurt as compared to fluid milk is the slower oral-caecal transit time of yoghurt. *Marteau* et al. (1990) used an intestinal perfusion technique to determine that only 20% of the lactase

activity in yoghurt reached the terminal ileum, and that 90% of lactose from yoghurt is digested in the small intestine of lactose intolerant subjects.

The improved digestion of lactose by lactase-deficient subjects has been demonstrated with a variety of commercial and laboratory-prepared yoghurts. *Wytock* and *DiPalma* (1988) showed that two of three commercial yoghurts resulted in significantly lower breath hydrogen levels than a liquid lactose control. Results with one of the commercial yoghurts were not statistically different from the control, suggesting that some commercial yoghurts are more effective than others. Since no lactase activities or starter culture population levels were conducted on the commercial yoghurts, it is not possible to determine the cause of the differences. In general, little attention has been paid to the strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* used in yoghurts tested, and no reports of strain optimisation for this application have been published. This may partly be because the relationship of internal lactase levels of different strains and their effectiveness at aiding yoghurt digestion is unclear. *Martini* et al., (1991b) tested four laboratory yoghurts prepared with different *S. thermophilus* and *L. bulgaricus* strains for their lactase levels and their ability to aid lactose digestion in lactase-deficient subjects. Although lactase levels differed substantially, the effect on breath hydrogen levels were not statistically different and were similar. Intuitively it would seem that selection of strains with high lactase levels would provide for optimal effectiveness, but other parameters may also be involved in delivery of lactase to the intestine. Important parameters may include: (1) the inherent resistance of lactase to stomach acid or intestinal bile, (2) the ability of specific strains to shield intracellular lactase from adverse conditions but allow contact with lactose when needed in the intestinal tract, or (3) inherent lactase activity under physiological conditions.

Testing of commercial unfermented milk products containing *L. acidophilus* cultures suggests that these products are not as effective as yoghurt in facilitating lactose digestion. Recent research suggests that this may be due, at least in part, to the lower level of culture added to these fluid products. In the USA, no fluid products currently are supplemented with yoghurt bacteria. Fluid products containing *L. acidophilus* or *Bifidobacterium* strains are available, but these bacteria do not appear to be as effective as yoghurt cultures and levels of these bacteria in fluid milk are much lower than yoghurt cultures in yoghurt (approximately 2 x 10^6/ml). Only two states, Oregon and California, regulate these levels; therefore no means of enforcement of minimal levels in other states exists. In contrast, high quality yoghurt generally has in excess of 10^8/ml each of *S. thermophilus* and *L. bulgaricus*. If adequate levels of yoghurt bacteria are provided in a fluid milk, this product becomes an effective aid for lactose digestion (*Lin* et al., 1991).

Specific differences in lactase delivery potential between yoghurt cultures and *L. acidophilus* exist. One hypothesis suggests that yoghurt cultures, permeabilised by bile in the intestine, more readily enable contact between ingested lactose and lactase. Bile-resistant *L. acidophilus* are not permeabilised in the intestine and lactose digestion is more regulated. Regardless of the mechanism, it appears that even at equivalent bacterial levels, *L. acidophilus* strains are not as effective as yoghurt strains in aiding lactose digestion *in vivo*, and among *L. acidophilus* strains, some are better than others (*Lin* et al., 1991).

Research also suggests that the role of yoghurt in facilitating the digestion of

lactose in lactose-intolerant people is more complex than simple lactase delivery. The evidence for this is the lack of linearity between lactase delivery and breath hydrogen results (*Martini* et al., 1991b). Total lactase levels in the yoghurt differed up to three-fold and specific lactase activities up to 2-fold, but no differences in *in vivo* lactose digestion were seen. The authors conclude that "All yoghurts dramatically and similarly improved lactose digestion, regardless of their total or specific ß-gal (lactase) activity". The delivery of lactase to the intestine is likely a function of several factors, possibly including survival of lactase through the stomach, level of contact between lactase and lactose in the intestine, and lactase levels in the yoghurt.

Many different aspects of yoghurt-mediated improvement of lactose digestion have been tested. Research suggests that bacteria delivered in yoghurt are not able to aid the digestion of lactose beyond that normally present in yoghurt (*Martini* et al., 1991a), that both *S. thermophilus* and *L. bulgaricus* strains individually can suitably mediate the effect, i.e. a mixed culture is not essential (*Lin* et al., 1991), and yoghurt with heat killed bacteria is not as effective as yoghurt containing viable lactic acid bacteria (*Savaiano* et al., 1984), but some improvement is seen (*Marteau* et al., 1990).

CONCLUSIONS ON CLINICAL STUDIES

Some studies focused on specific clinical conditions suggest that probiotics can have a positive influence on health. Perhaps the most conclusive evidence is for aiding the digestion of lactose. Also, good evidence exists for some anti-diarrhoeal effects of some probiotics given in high enough doses. The ability of probiotic cultures to alter metabolic activity of faecal flora has been supported by several lines of research. However, it is not clear to what extent these results relate to improving health of healthy adults.

The clinical results generated so far also suggest that adherence is not required for the observed effects. Any effects seen appear to require high numbers of viable bacteria fed on a regular basis. Cessation of feeding correlates with an absence of effect.

SAFETY CONCERNS

Efforts to substantiate clinical effectiveness of probiotic lactobacilli and bifidobacteria in humans have resulted in exploration of clinical situations where their benefits may be revealed. Many clinical trials have been conducted, some with healthy subjects and some with clinically-compromised patients. The safety of this approach has been justified since lactic acid bacteria are considered rarely pathogenic to man. Although the safety of fermented food products containing large numbers of traditional lactic acid bacteria is unquestioned, supported by centuries of safe consumption, human consumption of large numbers of lactic cultures of intestinal origin is a more recent development (*Driessen* and *de Boer*, 1989). Intestinal lactobacilli and bifidobacteria have been included in food products in more recent years. Consumption patterns of these products are geographically distinct, but Japan and some

countries in Europe have shown high per capita levels of consumption for at least a decade, with no ill effects.

From the backdrop of a long history of safety, however, comes isolated medical reports of association of some lactic acid bacteria with human infection. *Aguirre* and *Collins* (1993) have recently reviewed this area. They cite publications documenting clinical infection in humans associated with *Lactobacillus* (68 cases), *Lueconostoc* (27 cases), and *Pediococcus* (18 cases) strains. *Torre* et al. (1990) reported necrotising pneumonitis caused by *Lactococcus cremoris* in a HIV-infected intravenous drug addict. Antibiotic treatment was successful and the patient recovered. The authors speculated that the organism infected the patient through the oropharynx, and originated from unpasteurised milk and cheese ingested by the patient. *Giraud* et al. (1993) report a fatal case of *Leuconostoc* infection in an adult bone marrow transplant recipient treated with vancomycin. The natural vancomycin-resistance of *Leuconostoc* species makes them a threat for these patients.

In evaluating the reported cases, it is clear that most of the patients involved with these infections are in a compromised health state, which leads some of these bacteria to express as opportunistic pathogens. No indications have suggested a potential problem with administration of high levels of these bacteria in clinical studies or in commercial products. However, it is also important to keep in mind that clinical trials using probiotics have also turned more recently to their effect on clinically-compromised patients. These experiments should be considered with caution. Furthermore, it should be recognised that food products containing these bacteria will be considered safe for general use, and therefore must be judged safe for all consumers, healthy or not. Although not all reported cases have definitively established the causative role of these lactic acid bacteria in human infection, *Aguirre* and *Collins* (1993) recommend that "the view that lactic acid bacteria are non-pathogenic merits reassessment in view of the increasing numbers of reports of their association with human clinical infection".

THE FUTURE

The future of probiotics in the diet of humans is largely tied to (1) successful research programs focused in this area and (2) responsible, thoughtful formulation of commercial probiotic products. Although many products are currently being marketed, and are promoted directly or indirectly with health claims, many of them deliver low levels of strains of questionable benefit to humans. Marketing products based on testimonial evidence alone does not impose reasonable scrutiny of product quality and efficacy. Since no quality standards exist for probiotic efficacy beyond, perhaps, viable population levels and purity, efficacy of commercial products is difficult to substantiate as a consumer. In the long run, products which do not meet with customers' expectations will not endure, and an overall negative impact will surely be seen for probiotic-containing products in general.

In the United States, it is now required that health claims made on foods comply with specific Food and Drug Administration (FDA) regulations, published as part of the newly enacted Nutrition Labelling and Education Act. Any health claims will be required to be approved by the FDA, a process which will require substantial scientific support for claims.

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CLINICAL EFFECTS OF BIFIDOBACTERIA AND LACTOBACILLI

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SUMMARY

To clarify the rationale of administration of *Bifidobacterium* to humans, we evaluated the faecal microflora in different age groups from the view point of colonisation resistance. Our findings suggest that the number of *Bifidobacterium* is the potential marker of the stability of the human intestinal microflora. On the clinical effects of *Bifidobacterium,* administration of *B. breve* preparation (BBG-01; 10^9 cells/g) to the patients with *Campylobacter* enteritis (n=133) showed the enhancement of eradication of *Campylobacter jejuni* along with the recovery of normal flora. In the cases of infantile intractable diarrhoea primarily induced by antibiotics (n=15, mean 2.5 yr.), the stool frequency and appearance were dramatically improved within 3 to 7 days after administration of BBG-01 from chronic watery diarrhoea (mean 25 days), with normal flora predominating resident *Bifidobacterium* or administered *B. breve*. On the clinical effects of *Lactobacillus casei* Shirota strain, oral administration of *L. casei* preparation (BLP: 10^{10} cells/g) is useful for the prevention of the recurrence of superficial bladder cancer: the 50% recurrence free duration was prolonged significantly by BLP treatment (n=23, 350 days) to 1.8 times that in control group (n=25, 195 days). The suppressive effect of BLP on the specific urinary mutagenicity derived from the ingestion of cooked meat was demonstrated in 6 healthy non-smokers. BLP administration for 3 weeks resulted in the decrease of urinary mutagenicity (6-67%, average 47.5%) compared to before administration. The blood pressure-lowering effects of oral administration of extract from *L. casei* (LEx) on the systolic blood pressure (SBP) were demonstrated in spontaneously hypertensive rats (SHR), widely used as an animal model for hypertension. Oral doses of 1 to 10 mg/kg of LEx yielded a significant decrease of SBP in the SHRs, but had no effect on normotensive rats. Clinical application of LEx in patients with hypertension is under investigation.

INTRODUCTION

In 1907, *Metchnikoff* launched the theory that the intestinal microflora exerts important influences on health and longevity. Although controversial, his theory was responsible for much subsequent scientific research regarding the role of fermented and culture-containing dairy products in health. Research performed with germfree animals and the introduction of improved anaerobic

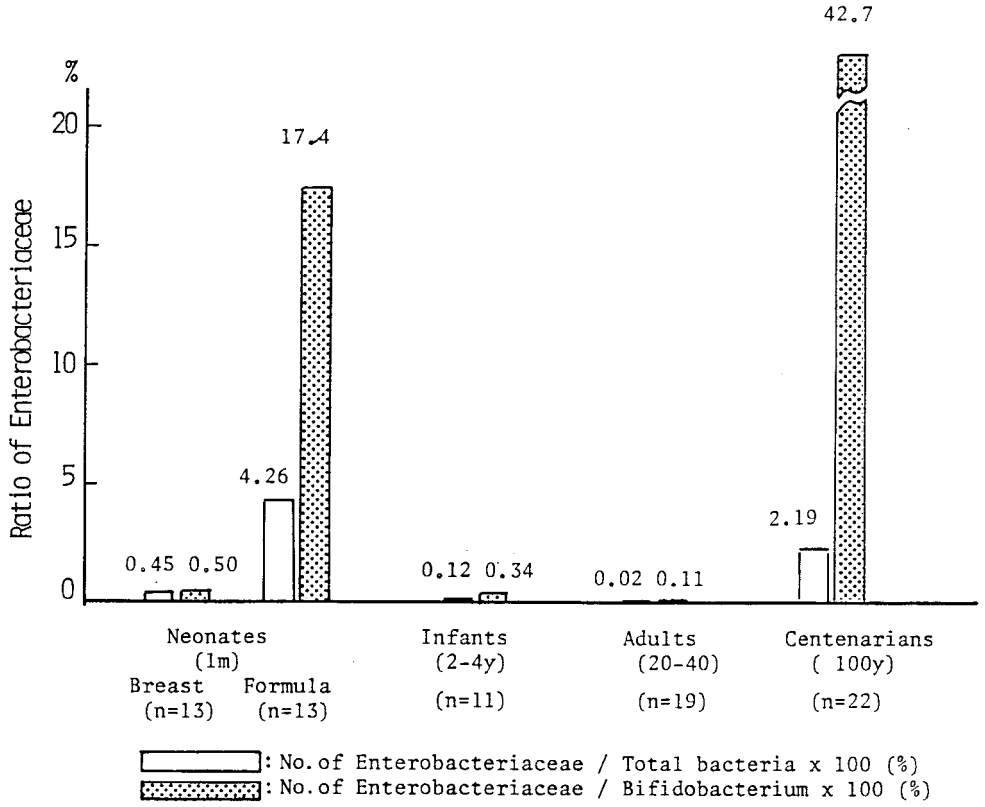

Figure 1: Colonisation resistance of human intestinal flora: the ratio of non-anaerobes to aerobes (from: *Mutai* and *Tanaka*, 1987).

culture techniques have been particularly useful in clarifying the significance of the interrelationships of diet and intestinal microflora in health and disease (*Mitsuoka*, 1982; *Finegold*, 1983; *Mallett*,1988). In spite of a rather large list of literature, the therapeutic effects of the preparations of bifidobacteria or lactobacilli as well as their culture-containing dairy products on human gastrointestinal disorders remain obscure or controversial. A criticism of most of the reports on the clinical effects of the administration of bifidobacteria or lactobacilli have been well summarised previously (*Conway*, 1989; *Renner*, 1991). The problems are:

1) not all the data reported in literature are based on well designed and controlled experiments and often lack statistical analysis, and

2) results obtained have to be evaluated based on the understanding of the specificity and stability of gastrointestinal ecosystem.

In view of this, the aim of this paper is to review the recent clinical effects of the administration of our Yakult strains of *Bifidobacterium breve* and *Lactobacillus casei* Shirota or their fermented dairy products in humans in a strictly controlled study.

RATIONALE OF ADMINISTRATION OF BIFIDOBACTERIA IN HUMANS

It has been recognised that breast-feeding protects infants from diarrhoea and a variety of respiratory infections which are major cause of infantile morbidity and mortality. Extensive studies have shown the significance of anti-infectious factors in breast milk, notably the maternal immune factors and the Bifidus factors. Much efforts have been made for the propagation of *Bifidobacterium* in bottle-fed infants either by the direct implantation of *Bifidobacterium* or adding Bifidus-growth promoting factors. In addition, it is well known that the normal intestinal flora is stable and prevents the colonisation of a number of environmental microbes including pathogenic bacteria. The protection against colonisation is involved in the natural resistance of humans to intestinal infections. *Van der Waaij* and colleagues (1972) proposed the concept of colonisation resistance and emphasised an important role for anaerobes in the maintenance of colonisation resistance in the intestines. In general, the ratio of non-anaerobes to anaerobes seems to be an indicator of the potential stability of the intestinal flora. Figure 1 shows the comparative values of the human intestinal flora in the different age groups according to our results on the faecal microflora of a group of healthy humans, consisting of 13 breast-fed and 13 formula-fed infants, 11 children, 19 adults, and 22 Okinawan centenarians, the world's reliable region on the geography of longevity (Table 1). The number of Enterobacteriaceae and total bacteria were used as the representatives of non-anaerobes and anaerobes, respectively. In addition, the number of Bifidobacteria, as the representatives of the most useful bacteria in the human intestine, was also used instead of total bacterial count. In the formula-fed infants and centenarians, the ratio of non-anaerobes to anaerobes was a factor of 10 to 100 higher than that of breast-fed infants, weanlings, and adults. Furthermore, when using the number of Bifdobacteria instead of total bacteria, these differences were more striking, suggesting that *Bifidobacterium* plays an important role in the maintenance of colonisation resistance. It is of interest that formula-fed infants and the aged are more susceptible to various infections than the other age groups.

EFFECT OF *BIFIDOBACTERIUM BREVE* ON INFANTILE DIARRHOEA

Diarrhoeal diseases are the major cause of morbidity and mortality in infants and young children, especially in developing countries. *Shigella*, enteropathogenic *Escherichia coli*, enterotoxigenic *E. coli*, *Campylobacter* and rotavirus are the most important aetiological agents (*Chen*, 1978). The gastroenteris due to *Campylobacter* and *Salmonella* are the most common cause of acute infectious diarrhoea in developed nations (*Chen*, 1978). In addition, the administration of broad-spectrum antibiotics often results in antibiotic-associated diarrhoea, the so called non-specific watery diarrhoea syndrome, and pseudomembranous enterocolitis due to *Clostridium difficile* (*Bartlett*, 1983).

Table 1: Composition of faecal microflora of healthy human in different age groups (from: *Mutai* and *Tanaka*, 1987)

Age group	Total bacteria	Bacteroidaceae	*Bifidobacterium*	*Clostridium* (L+)	Enterobac-teriaceae	*Enterococcus*	*Lactobacillus*	*Staphylococcus*
I: neonates (1 m) breast-fed (n=13)	10.77±0.33	9.31±0.24 (100)	10.72±0.38 (100)	7.25±2.58 (16)	8.42±0.91 (100)	6.78±1.73 (100)	7.05±1.36 (64)	6.15±1.58 (93)
II: neonates (1 m) formula-fed (n=13)	10.64±0.39	9.84±0.60 (100)	10.03±0.55 (85)	6.87±2.65 (16)	9.27±0.72 (100)	8.35±1.33 (100)	6.77±1.89 (77)	5.70±1.12 (100)
III: infants (2-4 y; n=11)	10.67±0.18	10.03±0.31 (100)	10.22±0.26 (100)	4.93±1.24 (91)	7.75±0.52 (100)	7.85±0.94 (100)	5.28±1.65 (100)	3.69±0.84 (100)
IV: adults (20-40 y; n=19)	10.98±0.28	10.72±0.32 (100)	10.30±0.32 (100)	4.17±1.56 (84)	7.33±0.78 (100)	6.67±1.11 (100)	6.24±1.71 (90)	4.10±0.92 (70)
V: centenarians (>100 y; n=22)	10.35±0.25	9.95±0.43 (100)	9.06±0.57 (86)	6.01±2.20 (91)	8.69±0.89 (100)	7.68±1.44 (100)	6.30±1.58 (91)	5.52±1.88 (77)
Significance								
I : II		**	**		*	*		
I : III		***	**		*		*	***
I : IV		***	**		***			***
I : V	***	***	***					
II : III					***			***
II : IV	**	***			***	***		**
II : V	*		***					
III : IV	**	***				**		
III : V	***		***		**			**
IV : V	***	***	***	**	***	*		*

Mean ± SD of log bacterial counts per gram faeces (%; the rate of occurrence).
Significant difference: *p<0.05; **p<0.01; ***p<0.001

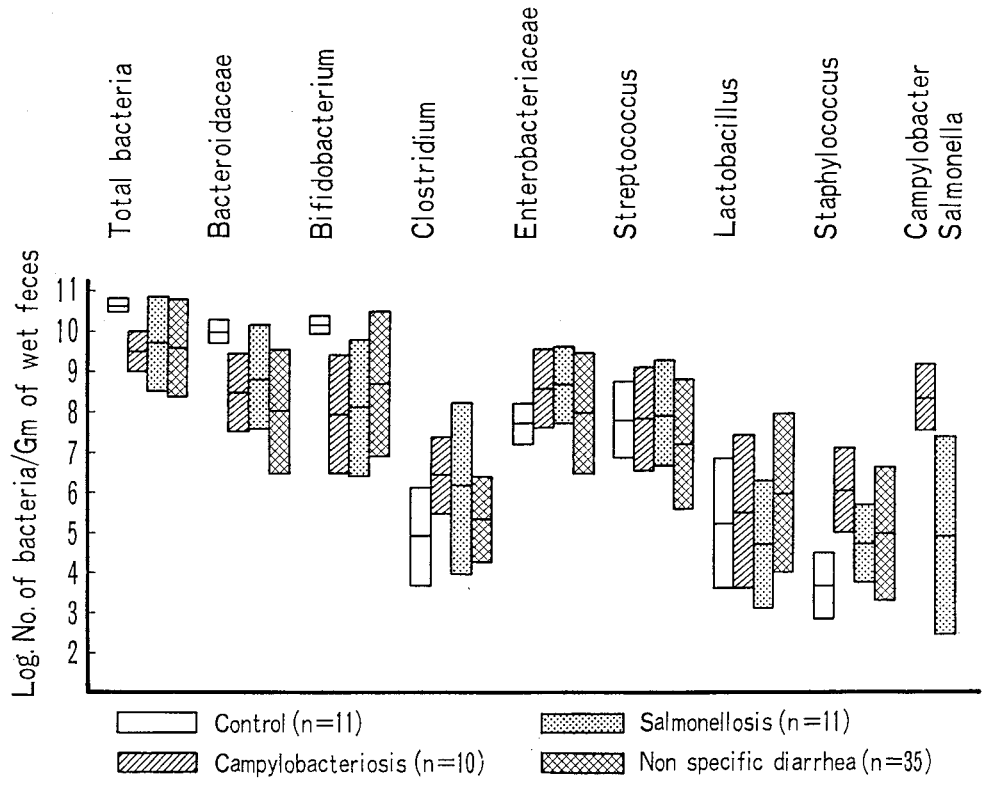

Figure 2: Faecal microflora of infants with diarrhoeal disease (from: *Tanaka* et al., 1989).

THE FAECAL MICROFLORA OF PATIENTS WITH ACUTE GASTROINTESTINAL INFECTIONS

In our institute, a total of 56 outpatients with acute diarrhoea at a modern University hospital in Tokyo were studied (*Tanaka* et al, 1990). Briefly, all faecal specimens were obtained by taking rectal swabs, and were transported to our institute in an anaerobic transport-medium. Medium preparation, dilution and inoculation were carried out anaerobically according to the modified VPI anaerobic roll tube methods. MVLG-KV (modified VL-G, MVLG, containing 80 µg/ml kanamycin and 1.0 µg/ml vancomycin, MPN, CW (Nissui, Tokyo) were used for counting total bacteria, Bacteroidaceae, *Bifidobacterium* and lecithinase positive *Clostridium,* respectively. The administered *Bifidobacterium breve* Yakult strain was enumerated using MPN *Bifidobacterium* selective medium containing streptomycin (3000 µg/ml) and neomycin (100 µg/ml). The numbers of Enterobacteriaceae, *Enterococcus, Lactobacillus* and *Staphylococcus* were determined using the selective medium of either DHL (Nissui), KMN or Staphylo No. 110 (Nissui). For the isolation of fungi, Candida GS medium (Eiken, Tokyo) was used. For the detection of enteropathogens, *Shigella, Salmonella* and *Yersinia* were examined using SS medium(Nissui), *Vibrio parahaemolyticus, Vibrio cholera* using TCBS medium (Eiken) and *Campylobacter* using Skillow's medium (Nissui). At

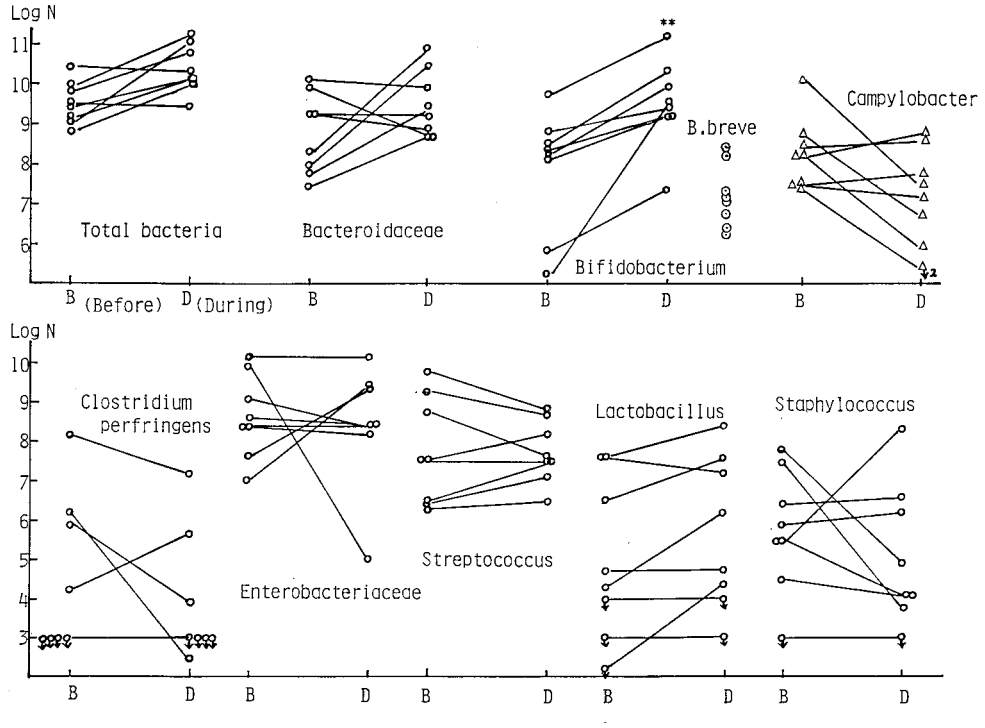

Figure 3: Effect of bacteriotherapy on the faecal flora of *Campylobacter* enteritis. Bacteriotherapy: administration of BBG-01 (*B. breve* preparation, 10^9 per day for 1 week; from: *Tanaka* et al., 1990).
**p<0.01 (Student's paired t-test)

least five strains of *Clostridium difficile* and enterotoxigenic *E. coli* were examined from each specimen using CCFA medium (Nissui) and DHL, respectively. The presence of toxin production was examined for each bacterium. Faecal cytotoxin and enterotoxin produced by *C. difficile* were determined by means of cytopathogenic effects (CPE) on HeLa cells and CPE neutralisation by anti-*Clostridium sorderii* antibody, kindly provided by Prof. Nakamura, School of Medicine, Kanazawa University. Enterotoxin was determined by means of a reversed passive latex agglutination kit (Denka Biological Laboratories, Tokyo). Heatlabile (LT) and heat stable toxin (ST) of enterotoxigenic *E. coli* were determined by a commercially available kit (Denka) and by intragastric administration in infant mice.

In the outpatients studied, the most commonly found enteropathogens were *Salmonella* (11 cases) and *Campylobacter* (10 cases) next to non-specific diarrhoea (35 cases). In the faecal microflora at the acute stage of diarrhoea, the number of anaerobes; total bacteria, Bacteroidaceae and *Bifidobacterium* were significantly reduced along with an increase of Enterobacteriaceae, *Staphylococcus,* and *Clostridium* (lecithinase positive), regardless the difference of the causative agents when compared to age-matched healthy infants (Figure 2).

EFFECT OF A *BIFIDOBACTERIUM BREVE* PREPARATION ON *CAMPYLOBACTER* ENTERITIS

Since it is shown that *Campylobacter* enteritis is the most common gastrointestinal infection, the effect of administration of a *B. breve* preparation, BBG-01 containing 3 x 10^9 cells/g, on the faecal flora of 8 patients with *Campylobacter* enteritis was investigated at first (Figure 3). During bacteriotherapy, the administered *B. breve* was recovered at a level of 10^6 to 10^8/g. In addition, the increase in number of total bacteria, Bacteroidaceae and *Bifidobacterium* was observed along with a decrease of *Campylobacter*. These results suggest that the administration of *B. breve* plays an important role in restoring the normal intestinal flora and thus shortens the time needed to eradicate *Campylobacter*.

In order to clarify in more detail the clinical effects of the *B. breve* preparation on *Campylobacter* enteritis, a total of 133 patients, aged 6 month to 15 years who had diarrhoea with *Campylobacter jejuni* alone, were used and randomly divided into three groups at the first visit to the hospital or clinic (*Tojyo* et al., 1987). Patients with mixed cultures of known gastrointestinal pathogens other than *C. jejuni* were excluded from the study. Briefly, the patients in group I (n=36) were treated with erythromycin (EM) at a concentration of 30-50 mg/kg daily in three divided doses for 7 days with additional antidiarrhoeal medication (Albumini Tannas and Alumini Silicas Naturalis). In group II (n=60), patients were given *B. breve* preparation (BBG-01) daily in three divided doses until *C. jejuni* was eradicated from the stool specimens. The additional antidiarrhoeal medication was also given in a similar manner as in group I. In group III (n=37), considered as the control group, patients were given only antidiarrhoeal medication until the symptoms were disappeared. In all patients, stool cultures were performed at least once a week until two consecutive cultures had become negative. Before bacteriotherapy, there were no differences between the three groups with regard to age, sex, maximum number of loose stools per day, blood in stool and fever (Table 2). The mean duration of diarrhoea was 2.7 days in

Table 2: Characteristics of patients with *Campylobacter* enteritis before treatment (from: *Tojyo* et al., 1987)

		I. Erythromycin n=40	II. BBG-01 n=60	III. Control n=37
Age:	< 2 yr.:	10	14	8
	2 - 5 yr.:	20	30	16
	> 5 yr.:	10	16	13
Sex (M/F):		22/28	34/26	24/13
Maximum number of loose stool/day (mean):		5.4	5.8	5.5
Blood in stool (%):		45	42	38
Fever (%):		50	57	51

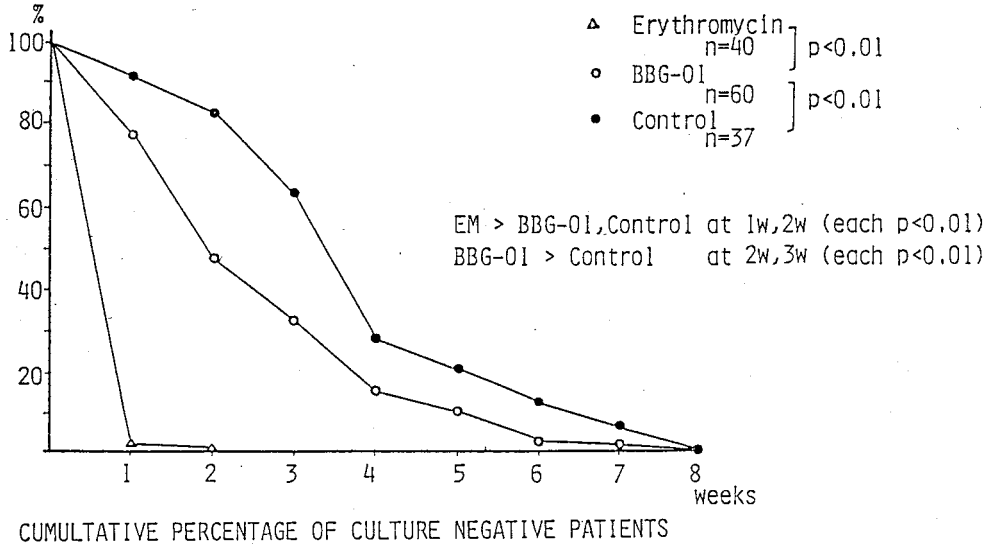

Figure 4: Effect of BBG-01 (*B. breve* preparation) on the eradication of *Campylobacter* (from: *Tojyo* et al., 1987).

the EM group, 3.2 days in the BBG-01 group and 2.7 days in the control group. The differences between these three groups were not statistically significant. On the effects of eradication of *Campylobacter jejuni*, as shown in Figure 4, EM is the most effective (EM>BBG-01, control, $p<0.01$), as was expected. However, BBG-01 is more effective than the control group (BBG-01>control, $p<0.01$). In addition, at weeks 2 and 3, the differences in the numbers of patients with a positive culture were also significant ($p<0.01$) between BBG-01 and control. In our study, EM did not alter the natural course of the disease in patients treated 7 days after the onset of symptoms, as has been reported by *Anders* and co-workers (1982). It was also demonstrated that the course of *Campylobacter* enteritis is self-limiting in normal children. Faecal carriage of *C. jejuni* persisted for up to 8 weeks if patients receiving no treatment and may be a source of contamination of the environment.

In summary, the administration of a *B. breve* preparation (BBG-01) was effective in eradicating *C. jejuni* and in restoring the normal intestinal flora. However, there were no differences in the duration of diarrhoea among three treatment groups (EM, BBG-01 and control antidiarrhoeal medication), regardless the different effects on the eradication of *C. jejuni*. This epidemiological result suggests that *B. breve* yoghurt may be useful for prophylaxis of infectious diarrhoea rather than for therapeutic use.

EFFECTS OF A *BIFIDOBACTERIUM BREVE* PREPARATION (BBG-01) ON ANTIBIOTIC-ASSOCIATED INTRACTABLE DIARRHOEA

A number of studies demonstrated that the suppression of the normal in-

Table 3: Summary of clinical responses of infants with intractable diarrhoea (from: *Hotta* et al., 1987)

Patient No.	Age	Sex	Underlying disease	Symptoms	Antibiotics	Bacteriotherapy	Duration of diarrhoea (days) before treatment	Duration of diarrhoea (days) after treatment
1	2y	F	periodic granulocytopenia	furuncle	CCL,CET,GM,PIPC	BLG-B	7	7
2	2y,10m	M		sepsis	KM,GM,ABPC,CBPC, CEZ,CMZ,CTX	BLG-B MTTMIL	30	14
3	1y	M	Kawasaki disease	bronchitis	CEX	MILMIL	35	7
4	1y	M		sepsis?	LMOX,CET	BBG-01	5	7
5	1y,8m	F	nephrosis	salmonellosis	ABPC,ST,KM,FOM	BLG-B	35	8
6	1m	M		bronchitis	ABPC,AMPC	BBG-01	25	7
7	15y	M	chronic nephritis	peritonitis	TOB,CET	BBG-01	11	6
8	1y,3m	M	Hirshsprung	sepsis?	TOB,CBPC,CTX,FOM GM,CAZ	BBG-01	10	3
9	1m	F	milk allergy	sepsis?	ABPC,CEZ,KM	BBG-01	25	7
10	3y	M	hemophilia B	sepsis?	CTX,CLDM,TOB	BBG-01	9	4
11	6y	M	hemophilia B	UTI	CTX,CXM	BLG-B	70	10
12	1m	M		sepsis?	ABPC,LMOX	BBG-01	7	4
13	3m	M	ventricular septal defect	sepsis	CTX,MCIPC,ABPC, LMOX,PIPC,CP	BBG-01	3-	10
14	4y,6m	F	Reye syndrome	broncho-pneumonia pulm. edema	ABPC,PIPC,CET,GM	BBG-01	40	7
15	3m	M		sepsis?	CMZ	BBG-01	40	4

BLG-B : Combined preparation of *Bifidobacterium breve* (10^9/g) and *Lactobacillus casei* (10^{10}/g).
BBG-01 : Preparation of *Bifidobacterium breve* (10^9/g).
MILMIL : Yoghurt containing 10^{10} of viable *Bifidobacterium breve*, *Bifidobacterium bifidum*, and 10^9 of *Lactobacillus acidophilus* per 100 ml.

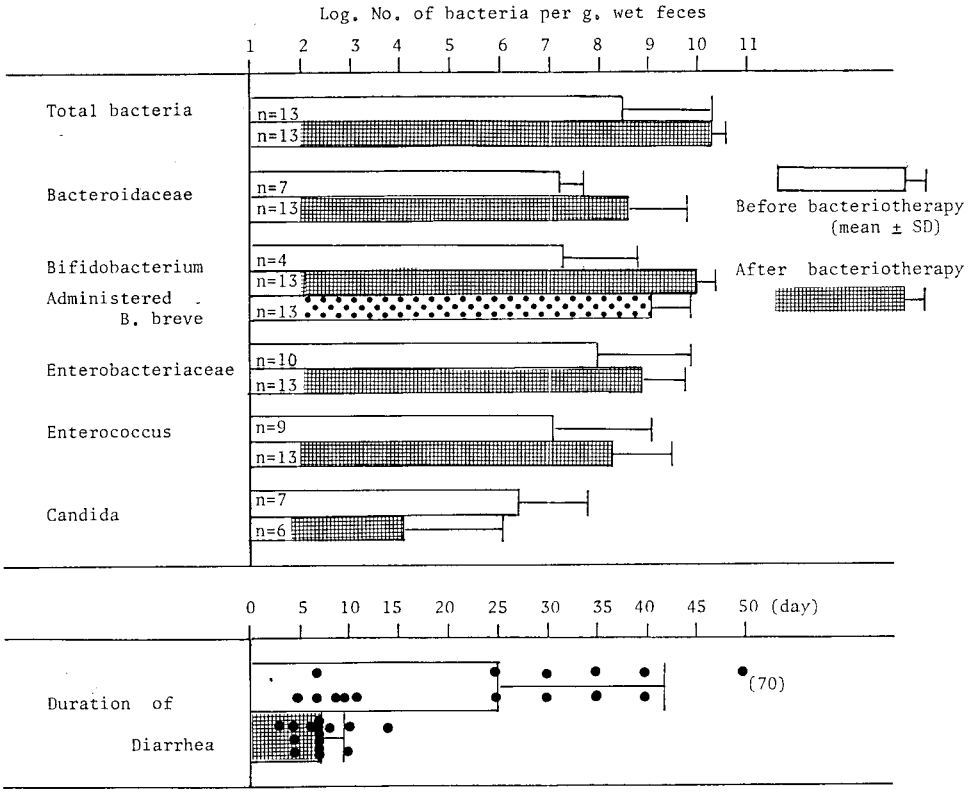

Figure 5: Effects of bacteriotherapy using *Bifidobacterium* on infantile intractable diarrhoea (from: *Hotta* et al., 1987).

testinal microflora leads to undesirable effects. It is well known, for example, that antibiotic therapy increases the risk of susceptibility to infections, bleeding problems and the selection of antibiotic-resistant strains. With regard to intestinal infections, the best known is pseudomembranous enterocolitis due to *Clostridium difficile.* Intractable diarrhoea in infancy is generally characterised by malnutrition, unresponsiveness to conservative therapy, and a high mortality. The most common definition of intractable diarrhoea in paediatric medicine was proposed by *Avery* et al. (1968):

1) diarrhoea continues for longer than two weeks,

2) occurs within three months after birth,

3) faecal cultures performed more than three times show no evidence of causative agents for the diarrhoea.

As for therapy, it is now recognised that administration of parenteral nutrients is the current basis for successful management of these infants.

We investigated the effects of the administration of the *B. breve* preparation (BBG-01) or commercially available *Bifidobacterium* yoghurt on infantile protracted diarrhoea primarily induced by antibiotic treatment (*Hoita* et al., 1987). The clinical features of the 15 patients are listed in Table 3. These patients (11 boys and 4 girls), ranging in age from 1 month to 15 years (mean 2.5 yr.), received antibiotic therapy for the treatment of extra-intestinal complaints such as septicaemia and respira-

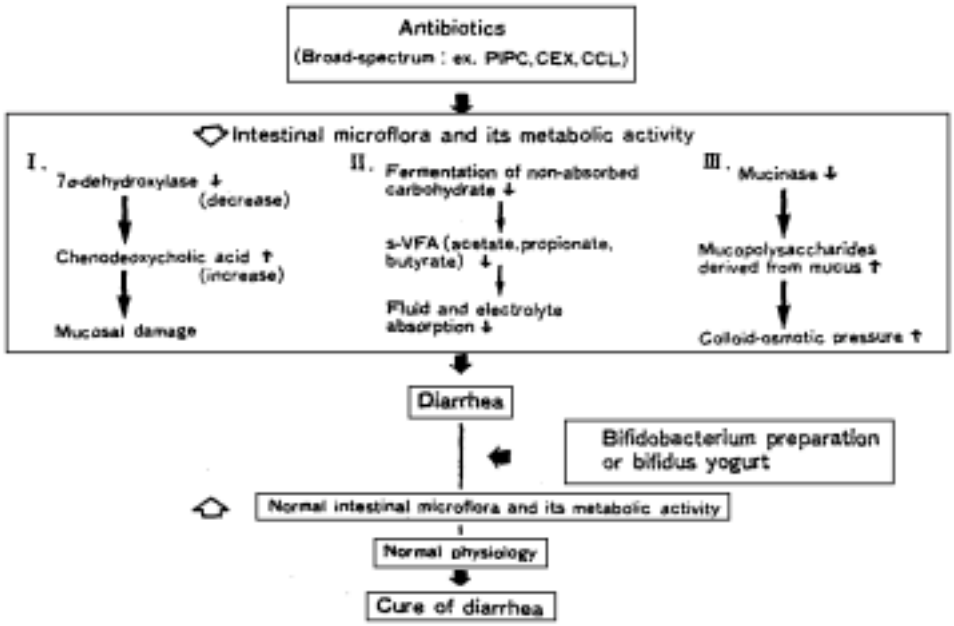

Figure 6: Some possible causes of antibiotic associated diarrhoea and cure mechanisms of *Bifidobacterium* therapy on infantile intractable diarrhoea (from: *Hotta* et al., 1987).

tory tract infections. The most frequently found infectious diseases in these patients were septicaemia (2 cases) and suspected septicaemia (6 cases), and respiratory infections (4 cases). Peritonitis, furuncles and *Salmonella* enteritis were found in one case each. The duration of diarrhoea prior to the onset of bacteriotherapy had an average of 25.3 days (ranging from 5-70 days) with deterioration of the general condition. In addition, conservative therapy (diet control, hyper-alimentation and chemotherapy) could not cure the diarrhoea. The antibiotics used included cephems, penicillins and aminoglycosides. Of the 15 patients, 10 patients received BBG-01; three combined preparations, BLG-B (*B. breve* at 10^9/g and *L. casei* at 10^{10}/g); the remaining two received either a combination of BLGB and Bifidus yoghurt (MILMIL) or Bifidus yoghurt alone containing 10^{10} cells of *B. breve*, *B. bifidum* and 10^9 of *L. acidophilus* per 100 ml. The intestinal microflora could be examined in 13 of the 15 patients. In most cases, an abnormal faecal flora was observed before bacteriotherapy; *Candida*, *Enterococcus* or Enterobacteriaceae often predominated with a marked decrease of anaerobes. *Bifidobacterium* was detected in only 4 of the 13 patients at 10^6/g wet faeces. During the disease, we could not detect any pathogens or toxins responsible for diarrhoea, such as *Clostridium difficile*. In all patients, the stool frequency and appearance dramatically improved within 1 week after oral administration of the *B. breve* preparation (Figure 5). After the diarrhoea was cured, the intestinal microflora of all the 13 patients studied normalised with a predominance of *Bifidobacterium* or administered *B. breve* at the level of 10^9 to 10^{10}/g. Figure 6 shows the possible mechanisms involved in the onset of antibiotic associated diarrhoea and in the recovery from diarrhoea with *Bifidobacterium*

Table 4: Characteristics of patients with superficial bladder cancer (from: *Aso* and *Akaza*, 1992)

		LC	Control	χ^2 test
Sex:	Male:	22	20	NS
	Female:	1	5	
Age (yr.):	<49:	0	2	NS
	50-59:	9	9	
	60-69:	6	8	
	>70:	8	6	
Stage:	pTa:	8	16	NS
	pTi:	14	8	
	pTx:	1	1	
Grade:	G1:	11	10	NS
	G2	12	15	
Papillary:		20	23	NS
Nonpapillary:		3	2	
Primary:		6	10	NS
Recurrent:		17	15	
Number:	Single:	5	4	NS
	Multiple:	18	19	
Size:	<1 cm:	17	17	NS
	1-3 cm:	6	7	
	>5 cm:	0	1	

bacteriotherapy. It is well known that the intestinal microflora has various metabolic activities that are essential for maintaining the normal physiological conditions of the host. Short-chain fatty acids (SCFA); acetic, propionic, and n-butyric acids are the major end products from unabsorbed carbohydrates of anaerobic microbial fermentation in the colon. It is reported that SCFA are rapidly absorbed and enhance the absorption of Na^+ and H_2O. Therefore, it can be said that the inhibition of anaerobes by antibiotic therapy probably reduces the luminal SCFA, resulting in malabsorption of H_2O and thus inducing diarrhoea. The administration of broad-spectrum antibiotics also did result in the decrease of bacterial 7α-dehydroxylation, which indicates inhibition of the conversion of primary bile acids to secondary bile acids. Thus, the increased concentration of primary bile acids, especially chenodeoxycholic acid may induce bile acid diarrhoea due to its detergent effects, causing injury to intestinal epithelial cells. Another possible explanation is the inhibition of bacterial degradation of mucopolysaccharides (mucin) secreted in the large intestine. The undegraded mucopolysaccharides may induce osmotic diarrhoea due to colloid-pressure induced fluid accumulation. This is suggested to be the pos-

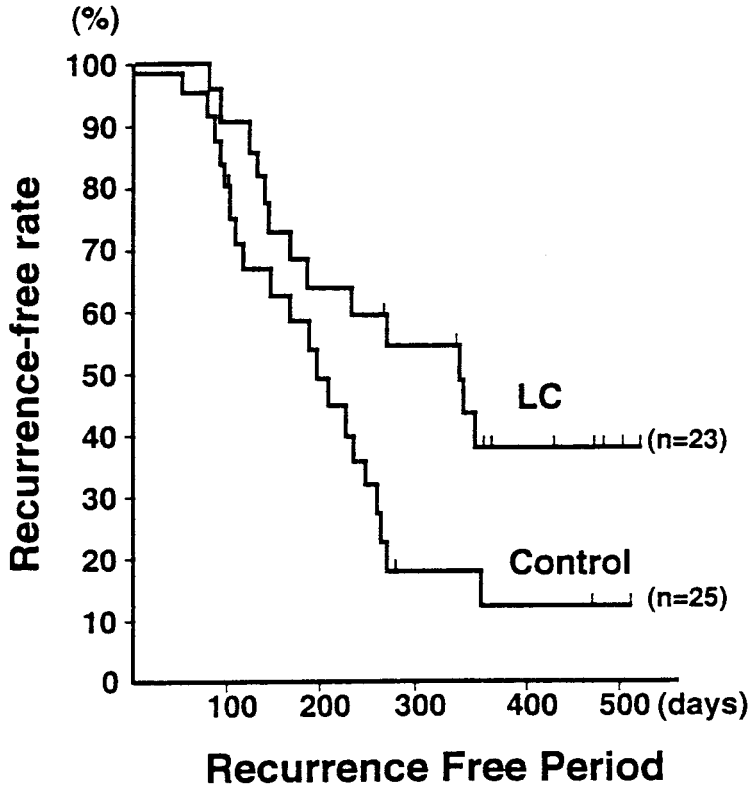

Figure 7: Effects of BLP (*L. casei* preparation, 10^{10} cells/g) on recurrence-free duration of superficial bladder cancer. The 50% recurrence-free intervals were 350 and 195 days for BLP and controls, respectively (Kaplan-Meier methods, *$p<0.05$; from: *Aso* and *Akaza*, a992)).

sible cause of caecal enlargement in germ-free rodents. In summary, malabsorption of water and electrolytes, probably induced by antibiotic therapy, may be corrected through normal metabolic activities such as SCFA production, bile acid metabolism and utilisation of mucin, resulting in a cure of diarrhoea. In summary, we stress the importance of the normal intestinal microflora, which plays an important role in the maintenance of normal physiology in the intestinal tract.

EFFECTS OF THE ADMINISTRATION OF *LACTOBACILLUS CASEI* ON HUMAN HEALTH

The beneficial role of *Lactobacillus* in the gastrointestinal tract has been one of the most controversial subjects within the area of gut microbial ecology (*Conway*, 1989). In the past decade, epidemiological and experimental studies suggested that there is an association between colon or breast cancer mortality and the so-called Western eating habit of consuming large amounts of animal protein and fat (*Wynder*, 1975, 1986). In this context, the more recent renewed interest in the nutritional significance of fermented dairy products has been fo-

cused on the possible preventive roles in colon and breast cancer through the improvement and stimulation of the microbial metabolic activity and immune system in the intestinal tract. In this section, the more recent evidences of the clinical effects of *Lactobacillus casei* on superficial bladder cancer, urinary mutagenicity and hypertension in humans will be reviewed.

Effect of *L. casei* on superficial bladder cancer

The prophylactic effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer was demonstrated by *Aso* and *Akaza* (1992). A high recurrence rate as well as adverse side effects of chemotherapy for superficial bladder cancer have been of great concern to urologists. A total of 58 patients with superficial bladder cancer enrolled in this study at 19 institutions, in order to study the preventive effects of *L. casei* preparation (BLP) on the recurrence after transurethral resection of the bladder tumour (TUR-Bt). The patients were randomised into a BLP-treated group (n=23) and a control group (n=25). In the BLP-treated group, patients received daily 3 x 10^{10} viable cells of *L. casei* for about one year until the completion of the follow-up period or until tumour recurrence. Control cases received no medication or placebo for BLP. During the study period, none of the patients received any other medication, including chemotherapeutic drugs, antibiotics, etc., that might affect the effects of BLP. Throughout the study, patients were examined by routine laboratory tests, including haematological examinations, blood chemistry and urinalysis. To detect the tumour recurrence, endoscopy was performed every three months after enrolment. Cytological examination of the urine and bladder biopsies were performed if necessary. The disease-free duration and recurrence-free rate were determined by the Kaplan-Meier method. There were no significant differences in patients characteristics between two groups (Table 4). As shown in Figure 7, the comparison of the disease-free duration between two groups revealed that the 50% recurrence-free interval in the BLP group was 350 days, which was 1.8 times that in the control group (195 days). In summary, the authors suggested that BLP has the potentiality to posses comparable efficacy in the prevention of the recurrence of the bladder tumour to that obtained with intravesical instillation of some chemotherapeutic agents. In addition, BLP has more advantages over the intravesical instillation, being less adverse side effects and the fact that it can easily be administered by oral route.

Effects of BLP on urinary mutagenicity

Many kinds of heterocyclic amines are formed in cooked or grilled meat and fish. These heterocyclic amines have been shown to be carcinogens in humans (*Wakabayashi* et al., 1992). Therefore, many investigators are now focusing on their potential cancer risks as dietary carcinogens in humans. More recently, a suppressive effect of a BLP on the specific urinary mutagenicity derived from the ingestion of cooked meat, was demonstrated by *Hayatsu* et al. (1993) using the Ames test. Briefly, administration of BLP was started immediately after the first confinement period and was continued for 3 weeks. During the periods of the first confinement "Before control", and the second confinement indicated "After *L. casei*", a total of 6 subjects aged 28 to 37 years were offered the ordinary Japanese meal with 10 gram of cooked beef which was heated on the pan at 170°C to 190°C for

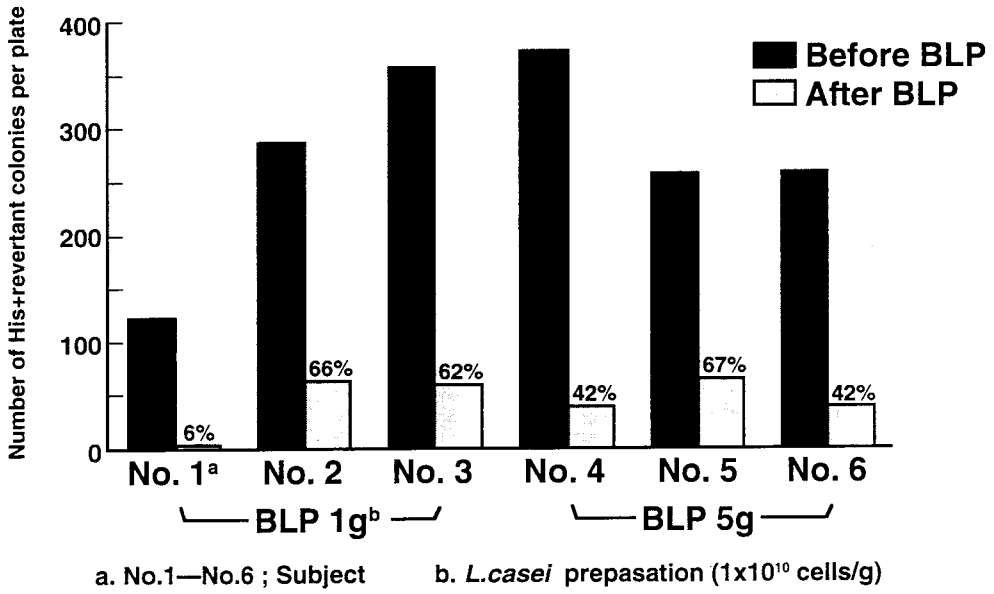

Figure 8: Effect of BLP (*L. casei* preparation) on beef meat-derived urinary mutagenicitiy (from: *Hayatsu* and *Hayatsu*, 1993).

10 min. The urinary mutagenicity was determined according to the Ames *Salmonella* test using *Salmonella typhimurium* TA98 with S9 mix. After BLP administration, the number of revertants decreased from 67 to 6%, compared to before BLP administration (Figure 8). There was a no difference in the suppressive effect of the BLP doses of 1 g or 5 g. The effective mechanisms are now under investigation in our institute, including the possibility of increased degradation of mutagens by the modified intestinal microflora as well as the mutagen binding by administered *L. casei.* In summary, the authors suggested that a decreased mutagen level in the urine by BLP administration may reflect a decrease in systemic mutagen exposure and may reduce cancer risks.

Effects of *L. casei* on hypertension

In our institute, the blood pressure-lowering effect of oral administration of the extract of autologous cell lysates from *L. casei* (LEx) on the systolic blood pressure (SBP) has been demonstrated in spontaneously hypertensive rats (SHR), a widely used animal model for hypertension. The clinical effects of LEx in patients with hypertension are now under investigation (*Hata* et al., personal communication). In brief, a total of 28 patients under treatment with anti-hypertensive drugs were randomised into two groups; given LEx (n=14) and a control group (n=14), fed dextrin as placebo. There were no differences in the clinical characteristics between the two groups. The LEx was administered orally at a dose of 800 mg per day for 2 months. The delta systolic blood pressure in patients given LEx was significantly lower than in the control group ($p<0.05$; data not shown). Antihypertensive compounds were purified from an extract of autrogous *L. casei* cell wall lysates (*Sawada* et al., 1990). The most effective compounds were polysaccharide-glycopeptide complexes, named SG-i, with a molecular

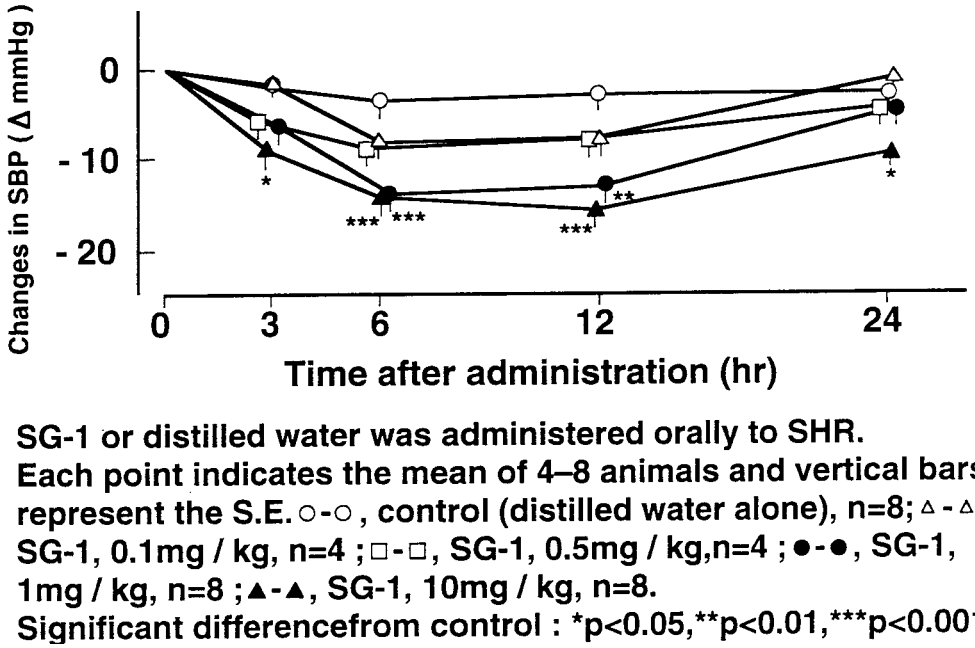

Figure 9: Effects of a single oral administration of SG-1 on the SBP of RHR (from: *Sawada* et al., 1990).

weight of about 180,000. The polysaccharide moiety of the complexes consisted of glucose, rhamnose, and galactose, whereas the glycopeptide moiety consisted of N-acetylglucosamine, N-acetylmuramic acid, asparagine, glutamine, alanine, and lysine. A significant decrease of systolic blood pressure was demonstrated at a dose of 1 to 10 mg of SG-1 per kg body weight (Figure 9). The mechanism of antihypertensive activity of SG-1 in the LEx is under investigation in our institute.

CONCLUDING REMARKS

In the past decade, there has increasingly been renewed and considerable interest in the ingestion of fermented dairy products containing viable *Bifidobacterium* and *Lactobacillus* to maintain a proper balance of normal intestinal flora and to enhance the beneficial relationship between host and intestinal flora. Although we are in a preliminary stage to understand the precise role of probiotics, scientific exploration of probiotic studies probably will help to develop feasible and practical measures to enhance the host's defence.

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MICROBIAL THERAPY WITH *ENTEROCOCCUS FAECALIS* AND *ESCHERICHIA COLI*: EXPERIMENTAL AND CLINICAL DATA

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SUMMARY

Microbial therapy with microbial preparations and autovaccines is a therapeutic method based upon experimental and controlled clinical studies recognised by social and private health insurances in Germany.

The treatment of various diseases ranging from acute and chronic infections in the ENT-field, in the respiratory tract, in the gastrointestinal area, in the urogenital tract, up to allergies and other, originally developed empirically and hardly understood from the conventional point of view, in these days wins comprehension via latest immunological insight. The embryologically, morphologically and immunologically conceivable mucosal immune system leads to a determinable basis of the broad spectrum of indications and opens new perspectives in therapy. Immunmodulation was in the centre of the scientific interest of a group at Herborn-Dill, Germany (Medical Association for Microbial Therapy), conducting numerous experimental and controlled clinical studies taking into consideration most recently measurable immunological parameters. Altogether, eleven randomised, double-blind and placebo controlled clinical trials were performed including a total of 2,334 patients. Hypotheses concerning efficacy were confirmed in animal experiments and in *in vitro* lymphocyte cultures of human volunteers. Different immunological signals intervene effectively in the complex network of the mucosal immune system, the immune system, the nervous system, the metabolism and the hormone system. Thus, forty years of careful medical observations in therapy with microbial preparations and autovaccines become validated.

FUNDAMENTALS

Immunological aspects

The basis of immunomodulatory therapies is the immune system of which we now know that it possesses a very much more complex structure than was originally assumed (*Roitt* et al., 1989). The immune system provides not only "immunity", i.e. protection from infection, but also serves for the performance of numerous other tasks in conjunction with the microflora, mucosae, metabolism, nervous system and hormone system. From the phylogenetic viewpoint, the intestine is to be regarded as the cradle of the immune system. The skin and especially the mucosae in the digestive tract are the direct borders with our environment so that it is quite logical to see a special immunological significance in these areas. Only recently,

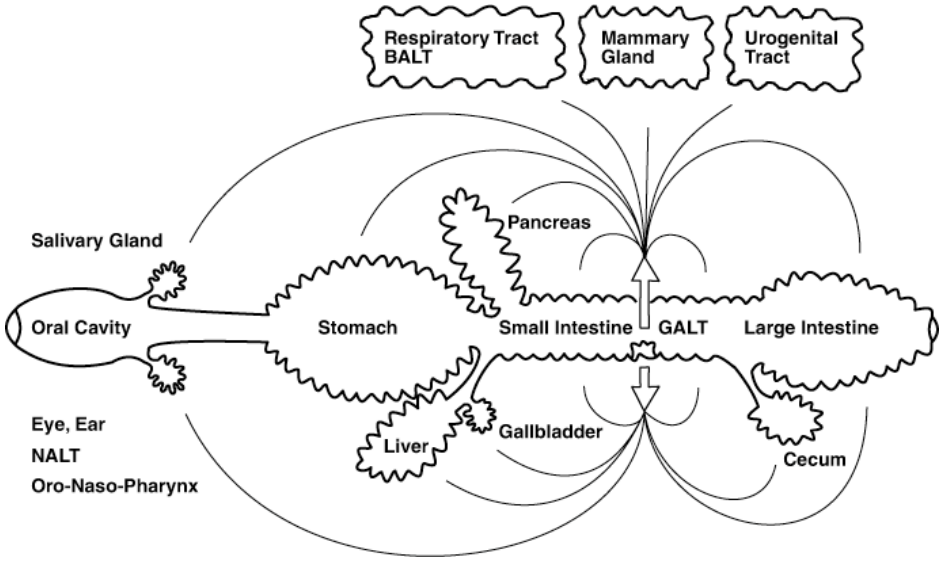

Figure 1: The mucosal immune system. Lymphocytes are sensitised at specific points of the mucosal immune system (e.g., in the lymphatic tissue associated with the intestine (GALT), move into the lymph capillaries and pass through the mesenteric lymph nodes, the thoracic duct and the blood stream (continuous arrows). These lymphocytes then migrate (interrupted arrows) to various mucosae of the gastrointestinal system and to mucosal tissues outside the intestinal tract (*Castro*, 1989).

it was learned, that about 85% of the immune system are to be attributed to the mucosae of the human organism. The morphological substrate of this relatively autonomous system are the organs developing from the entoderm in ontogenesis. It was first realised that there is an independent lymphatic tissue associated with the intestine (GALT). The latest findings have indicated that the mucosal immune system is common to all the mucosae, that the most diverse organs are linked with each other (*Gemsa* et al., 1991; *MacDonald* et al., 1990; *Ogra* et al., 1994; *Stern*, 1992). These concepts are shown in Figure 1. The boundary areas represented in the mucosal immune system cover an exceptionally large area: 400 m^2 small and large intestine, 80 m^2 lung (2.5 m^2 skin). A large part of these mucosae is normally colonised by microorganisms (*Fuller* et al., 1995; *van der Waaij* et al., 1990).

Microbiological aspects

Human beings are colonised by a fantastic variety and number of microorganisms. Microbe populations of different compositions in different densities colonise different habitats: in the digestive tract (mouth, nasal and pharyngeal tract, stomach, small intestine, large intestine), the vagina, while also the skin is populated by microbes. The small intestine and the large intestine alone are colonised by about 300 to 500 different species of microbes that can be divided into 17 families and 47 genera. From intensive studies on germfree animals, very much was learnt about the significance of the microflora, especially concerning the structure and the functions of the immune system. In germfree animals, 85% of the immune system, i.e., the mucosal immune system, is not developed at all. The number of granulocytes is substantially reduced and the function of these granulocytes is

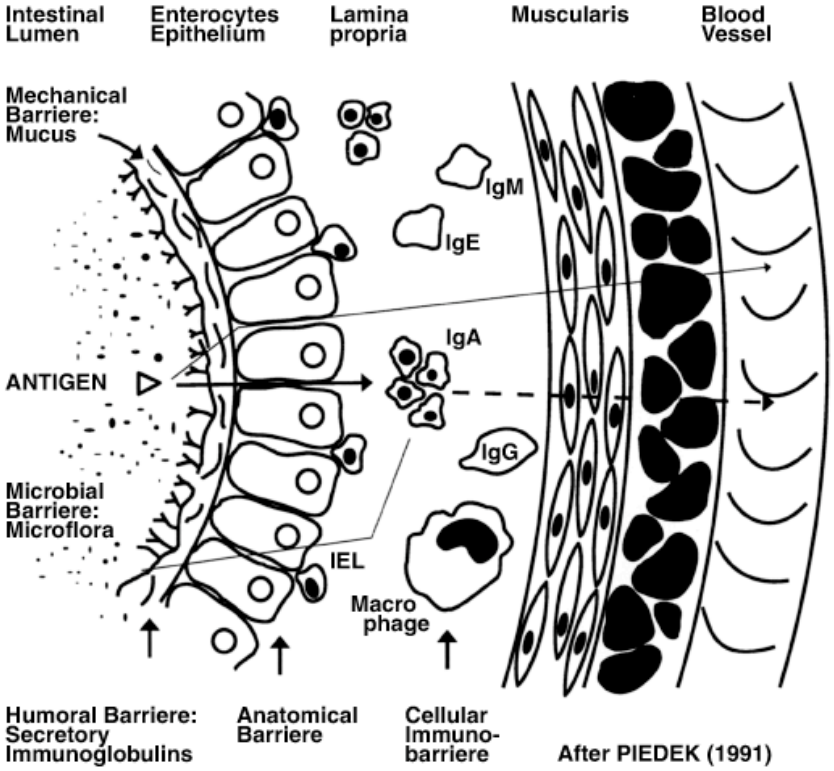

Figure 2: Graphical representation of the barrier function of the mucosal immune system on the example of the intestinal mucosa. IEL = intraepithelial lymphocytes (*van der Waaij* et al., 1995b).

limited. Relations between the microbes were studied by inoculation of germfree animals with microorganisms and it was experienced that the complex microbial populations stabilise in equilibria that resist colonisation by invading microbes from outside. This resistance is called colonisation resistance. Therefore the normal microflora is of decisive importance for the structure and the functions of the mucosal immune system. In addition, the normal microflora on its own provides a certain protection against pathogenic invaders. The microflora is thus an extremely important factor for health or disease. In the arrangement of the defence devices in most of the mucosae, the normal microflora represents one of the first defensive barriers (Figure 2) (*Fuller* et al., 1995; *van der Waaij* et al., 1990).

Symbiosis

Anton Heinrich de Bary defined the term "symbiosis" in 1879: "Symbiosis is the persistent and intimate living together of dissimilarly named organisms. Parasitism is the most popular and most exquisite phenomenon of symbiosis". Despite early misinterpretations and still existing misuse of the original meaning is accepted in modern biological sciences, as expressed during a Symposium of the Society for Experimental Biology in Cambridge by Starr in 1975: "'Symbiosis' is an eminently appropriate term. But an unfortunate second usage, in which the term 'symbiosis' has been limited to those organismic associations that are mutually beneficial, has crept in. It is high time to reverse the semantic deterioration. I have resolved, not only to use and to foster the use of the term

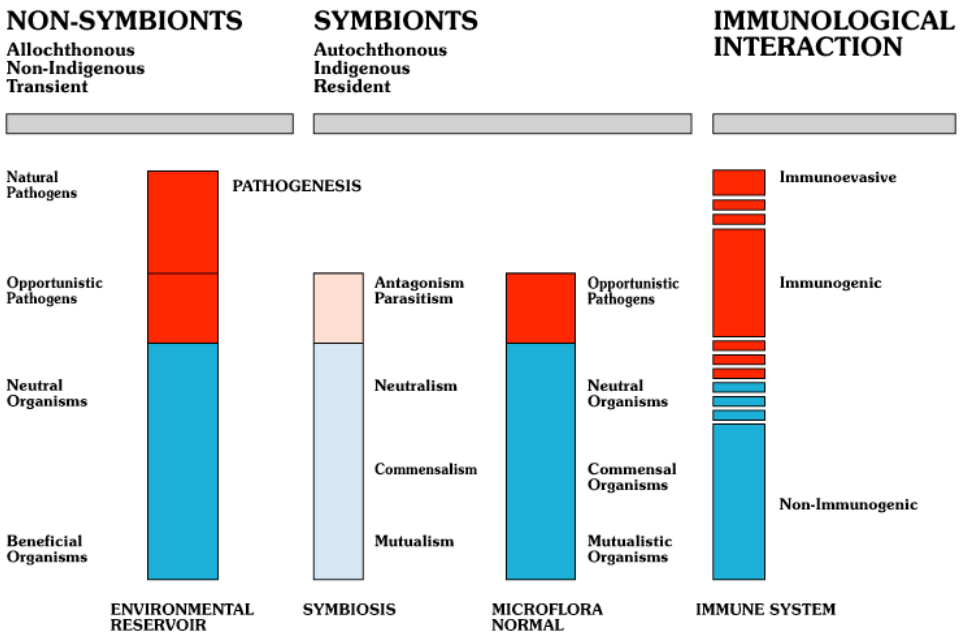

Figure 3: Microflora populations in the digestive tract (*Rusch*, 1989).

'symbiosis', but also to have it mean pretty much what de Bary intended." The definition of symbiosis covers a wide span of associations, including parasitism, neutralism, commensalism and mutualism. "The value of the concept resides in the widening of the concept of organism as structural unit to include heterogeneous systems as a "functional unit" or "functional field" (*Gregory*, 1951; *Rusch*, 1989).

This concept was transposed by an international study group to the consideration of microflora populations in the digestive tract (*Rusch*, 1989). The resulting classifications are represented in Figure 3. Completely in the sense of de Bary, man and microbes are understood as a symbiotic unity. The "normal" human microflora also contains opportunistic microorganisms with which humans live together in persistent and intimate partnership, microbes, normally under the control of neutral, commensal and mutualistic microorganisms and therefore cannot develop their pathogenic properties. Figure 4 is constructed on the concepts shown in Figure 3 and illustrates possible therapeutic consequences (*Rusch*, 1989).

MICROBIAL THERAPY

Definition

Microbial Therapy is defined as the oral and parenteral use of different live and/or killed microbes and/or their constituents for therapeutic purposes. The essential elements of Microbial Therapy are microbial preparations and autovaccines. About 35 microbial preparations are available in Germany (*Kolb* and *Maaß*, 1991). In spite of the tremendous variety of species of symbiotic microbes, essentially only four species are the basis for most preparations. *Enterococcus faecalis*, *Escherichia coli*,

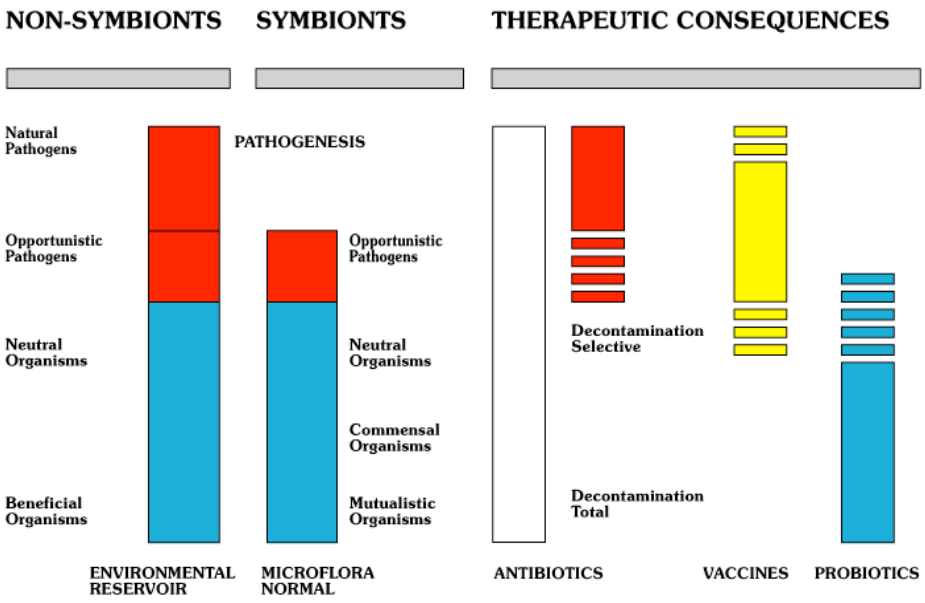

Figure 4: Therapeutic consequences in relation to the classification of symbionts and non-symbionts. (*Rusch*, 1989).

Bifidobacterium bifidum and lactobacilli. For non-symbionts, *Saccharomyces boulardii* are used.

In the context of therapy with microbial preparations, there exist terms such as "symbiosis treatment" (with several widely different interpretations), "therapy of dysbiosis", "microflora regulation", "microecological therapy" and similar connotations and terms reflecting obsolete views or which have little scientific background and are controversial. Microbial Therapy is to be clearly distinguished from these various therapy concepts (*Kolb* and *Maaß*, 1991).

Mode of action

Already in the seventies, the Medical Association for Microbial Therapy was confronted with the mode of action of microbial preparations and autovaccines. At the time, possible parameters for the determination of the efficacy were: flora modulation, modulation of metabolic activities of the host and immunomodulation. Because of major diagnostic problems and lacking clinical relevance, the flora modulation parameter was discarded. For similar reasons the metabolism parameter faded into the background.

Experimental and clinical studies

During the second half of the 1970's, the rapid advance in the knowledge of immunology led to a number of experimental studies in the USA to establish the foundations for later clinical tests. In the 1980's, several clinical trials were conducted on this basis. Two of these studies may serve as examples. On 106 patients with chronically recurrent disease of the upper respiratory tract, a randomised double-blind placebo study was conducted with the oral administration of an enterococcal preparation. Details of this study were published (*Kalinski*, 1986, 1987; *Rosenkranz* and *Grundmann*, 1994; *Rusch* et al., 1986; *Rusch*, 1987). The clinical results of this study are illustrated in Figure 5. After a treatment of three months, a significant

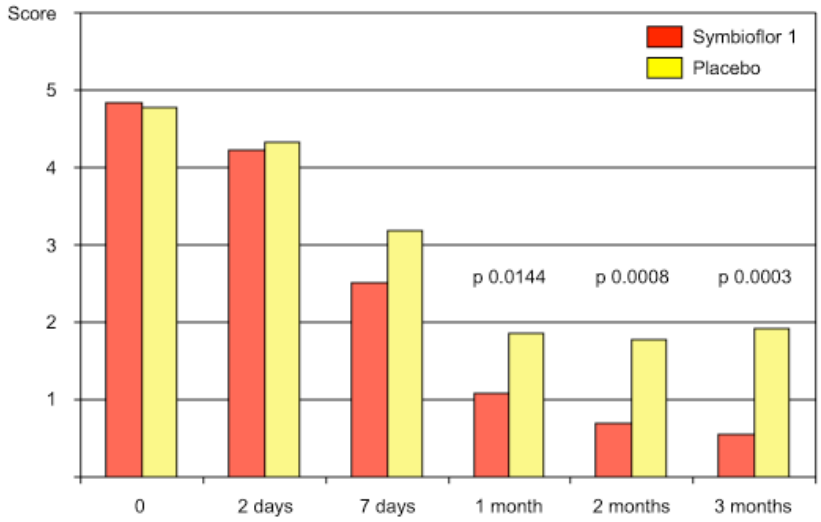

Figure 5: Clinical results (summarised in a score of clinical findings: the higher the score, the worse the findings) of a study with *Enterococcus faecalis* preparations (*Kalinski*, 1986, 1987; *Rosenkranz* and *Grundmann*, 1994; *Rusch* et al., 1986; *Rusch*, 1987).

difference between the verum and the placebo group was established. From the clinical point of view, the outcome of the study was very satisfying. Regarding the possible influence on immunological parameters, the evidence of corresponding tests were less satisfactory. Although the determination of serum immunoglobulins showed significant differences between the beginning

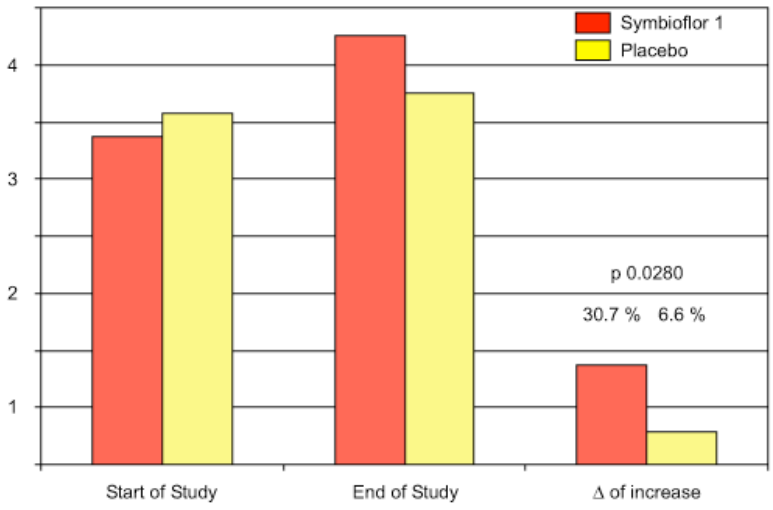

Figure 6: Evaluation of cellular immunity (Multitest Merieux) in the context of a clinical study with *E. faecalis* (see Figure 5). The increase of reactivity is significantly higher in the verum group than in the placebo group (*Kalinski*, 1986, 1987; *Rosenkranz* and *Grundmann*, 1994; *Rusch* et al., 1986; *Rusch*, 1987).

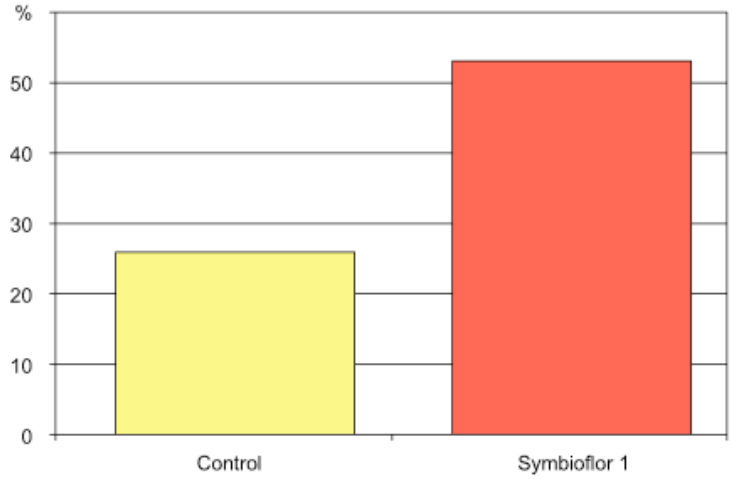

Figure 7: Determination of the granulocyte activity of C57Bl mice after three weeks of oral administration of an enterococcal preparation: Demonstration of the rate of intracellular killing of *Staphylococcus aureus*. The observed difference between the group of treated and untreated animals is significant (*Kalinski*, 1986, 1987; *Rosenkranz* and *Grundmann*, 1994; *Rusch* et al., 1986; *Rusch*, 1987).

and end in the treated group in some instances, these changes are within the limits of the immunological norm. However, in cellular immunity it was possible to demonstrate a remarkable difference between the two groups (Figure 6). With the help of the Mérieux Multitest skin test it was found that the increment of the increase in cellular reactivity was significantly

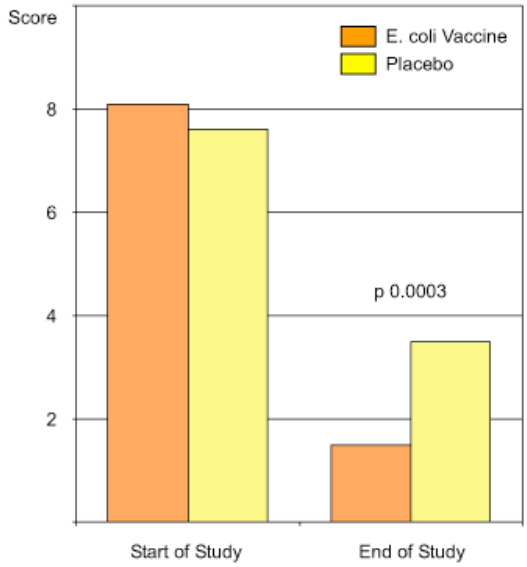

Figure 8: Clinical results (summary of clinical findings in a score, the higher the values, the worse the findings) of a study with *E. coli* vaccine for the treatment of chronic sinusitis (*Kalinski*, 1986, 1987; *Rosenkranz* and *Grundmann*, 1994; *Rusch* et al., 1986; *Rusch*, 1987).

Table 1: Randomised double-blind placebo-controlled clinical trials with microbial preparations (statistical analysis according to FDA-criteria).

Enterococcus faecalis* - live, oral					**Publication**
01	Tonsillitis	n = 160	significant	1986	published
02	Upper respiratory tract infections	n = 106	significant	1987	published
03	Immune status	n = 42	significant	1991	in preparation
04	Bronchitis*****	n = 140	significant	1991	in preparation
05	Sinusitis*****	n = 140	significant	1991	in preparation
06	Tonsillitis*****	n = 200	open	1991	in evaluation
07	Tonsillitis*****	n = 200	open	1991	in evaluation
08	Acute non-specific enteritis	n = 774	open	1992	in evaluation
***E. coli* and *E. faecalis* - killed, oral** **					
09	Irritable colon	n = 299	significant	1989	published
E. coli* - live, oral **					
10	Irritable colon	n = 299	significant	1989	published
E. coli* - killed, parenteral* **					
11	Sinusitis	n = 114	significant	1991	published
11 (total number of studies)		*2,334 (total number of patients)*			

* Symbioflor 1.
** Pro-Symbioflor.
*** Symbioflor 2.
**** Symbioflor-Antigen.
***** Determination of the PBMC-activity under evaluation.
Publications: see *Kalinski* (1986, 1987), *Rosenkranz* and *Grundmann* (1994), *Rusch* et al. (1986), and *Rusch* (1987) for quotations.

higher under verum than with placebo. In the middle of the 1980's, the routine clinical immunological diagnostics were still very limited, so clear statements could only be obtained from studies on animals. An example of this is shown in Figure 7.

The second element of Microbial Therapy are autovaccines. As placebo-controlled studies cannot be conducted with autovaccines, microorganisms, as they are usually employed by the Herborn group for the production of autovaccines, were pooled and used as hetero-vaccines. These *E. coli*-vaccines were used in a randomised double-blind placebo study on 114 patients with chronic sinusitis. The results are shown in Figure 8. Clinically significant results were achieved but with the techniques of that time did not lead to relevant immunological evidence. With the same vaccines, studies on animals were also conducted with significant results (*Kalinski*, 1986, 1987; *Rosenkranz* and *Grundmann*, 1994; *Rusch* et al., 1986; *Rusch*, 1987). It may seem paradoxical that an immunological signal from the colon (*Escherichia coli*!) is used in the treatment of an indication as sinusitis located far away from the colon - the mucosal immune system is the link. Details of this are given below.

This paragraph contains an overview of the so far conducted clinical tests of the Herborn study group. The overview

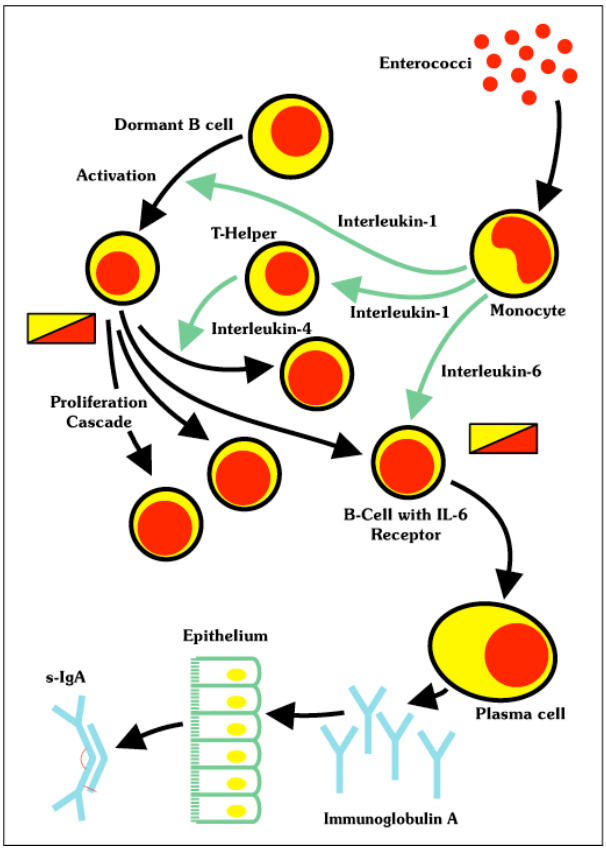

Figure 9: Schematic representation of a hypothesis on the efficacy of microbial preparations (*E. faecalis*). Enterococci stimulate monocytes/macrophages to produce interleukin-1ß and interleukin-6. This stimulates the activation and proliferation of B-cells with subsequent IgA and s-IgA production (*Gemsa* et al., 1991; *Kalinski*, 1986, 1987; *Kolb* and *Maaß*, 1991; *Rosenkranz* and *Grundmann*, 1994; *Rusch* et al., 1986; *Rusch*, 1987).

is given in Table 1. Based on the experience of studies conducted in the second half of the 1980's, several further studies were performed at the beginning of the 1990's. In eleven randomised double-blind and placebo-controlled studies, 234 patients were involved. All the studies have been concluded: three of them are still in the evaluation phase. Eight of the studies yielded clinically significant results. The statistical analysis was carried out according to the criteria of the US Food and Drug Organisation. With the results of these studies, the criteria for the determination of efficacy have been observed. The following remarks concern the immunomodulatory aspects.

IMMUNOMODULATION

Hypothesis

In a series of preliminary trials with lymphocyte cultures of several volunteer groups, it was found that cytokine activities can be stimulated by microbial preparations. In more recent clinical trials, based on the results of these examinations, lymphocyte cultures of the

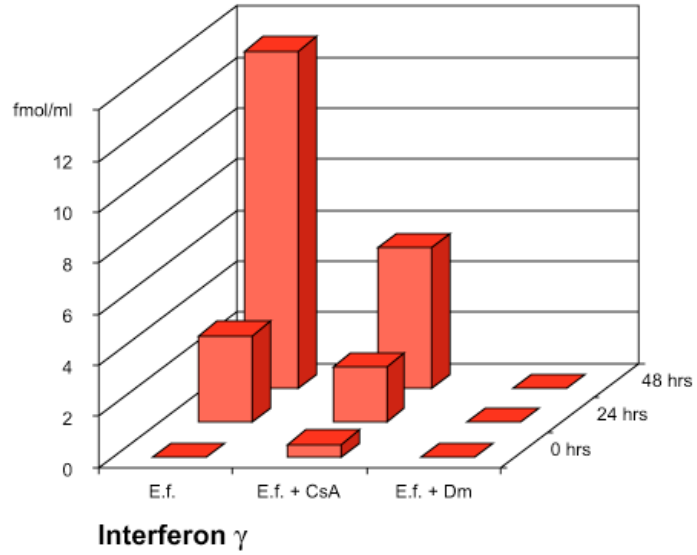

Figure 10: Stimulation of the release of gamma-interferon with *E. faecalis*. The release is inhibited by cyclosporin A (CsA) and dexamethasone (Dm) under simultaneous addition of *E. faecalis* (E.f.) to the incubation medium (*Rosenkranz* and *Grundmann*, 1994).

patients involved were also produced and the cytokine activities determined. The results are currently the subject of statistical analysis (see Table 1). The results of the preliminary trials led to the formulation of a hypothesis on the efficacy of microbial preparations.

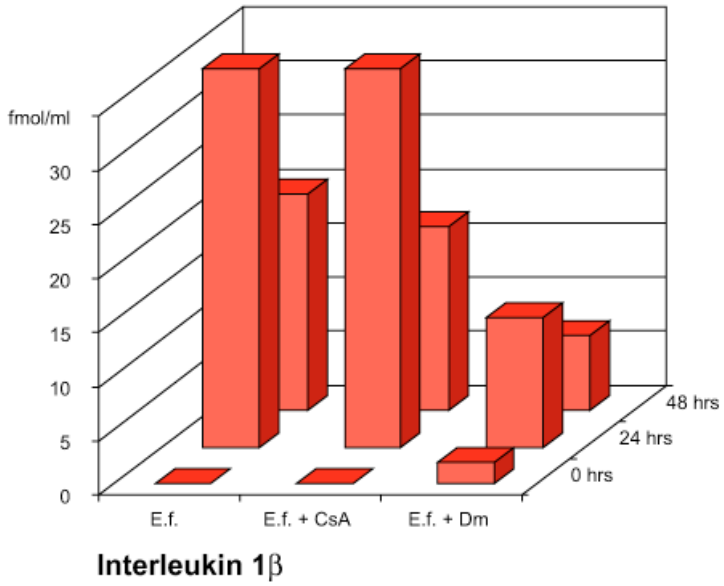

Figure 11: Release of interleukin-1ß due to stimulation with *E. faecalis* (E.f.). The release is inhibited by dexamethasone but not by cyclosporin A (*Rosenkranz* and *Grundmann*, 1994).

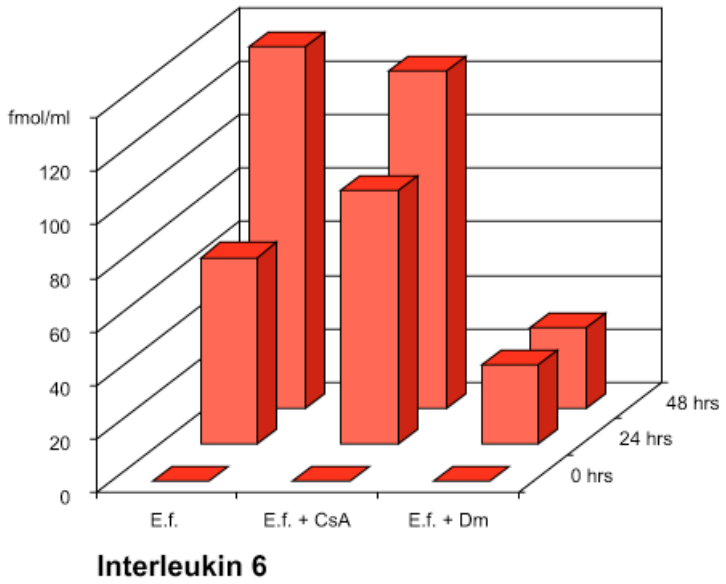

Figure 12: Release of interleukin-6 due to stimulation with *E. faecalis* (E.f.). The release is influenced by cyclosporin A (CsA) and inhibited by dexamethasone (Dm) (*Rosenkranz* and *Grundmann*, 1994).

Data

The hypothesis represented in Figure 9 was tested in two trial approaches. On the one hand, the effect of *Enterococcus faecalis* on the formation of cytokine was investigated in *in vitro* experiments with human peripheral, mononuclear blood cells; on the other, it was studied with mini-pigs whether *Enterococcus* leads to the stimulation of immunocompetent cells after oral administration and whether the mucosal system is stimulated. The *in vitro* tests on cell cultures with mononuclear peripheral blood cells of healthy volunteers prove the following effect of *Enterococcus faecalis*:

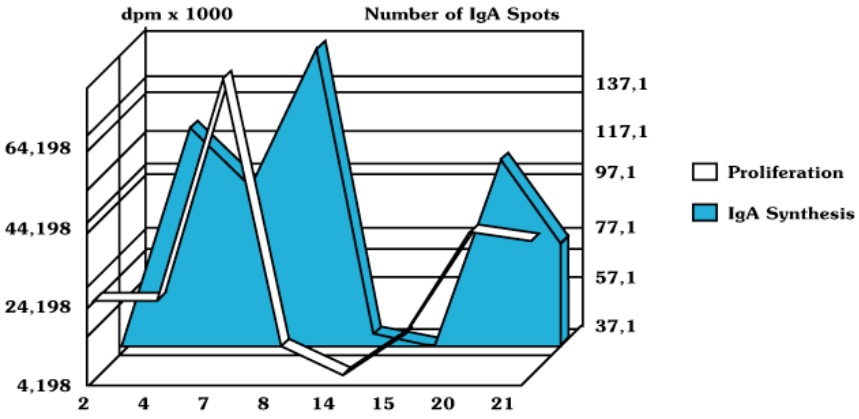

Figure 13: Comparison of the proliferation rate of immune cells in mini-pigs orally administered with *E. faecalis* and the IgA production in the Elispot-assay (*Ottendorfer*, 1994).

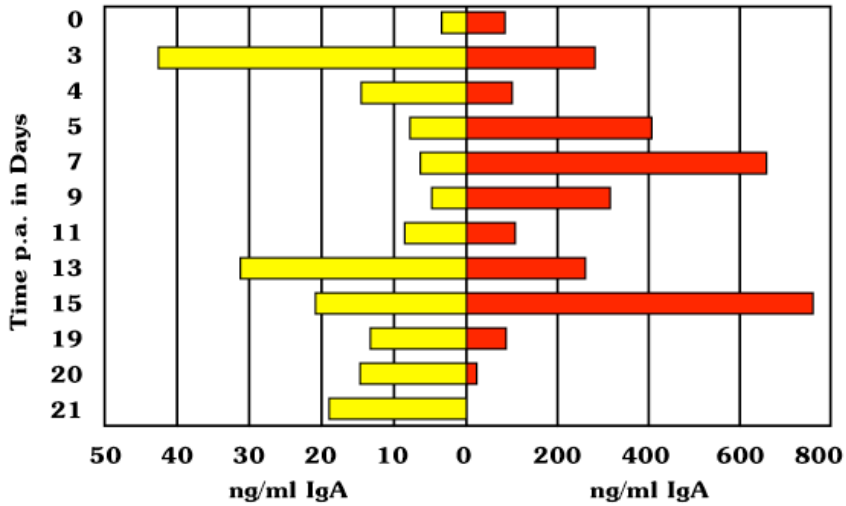

Figure 14: Concentration of total IgA in the saliva of mini-pigs during the administration of *E. faecalis*. The highest and lowest values are presented (*Ottendorfer*, 1994).

1. The release of interleukin-1ß and interleukin-6 is stimulated directly and dose-dependently. The release can be inhibited with dexamethasone; with cyclosporin A no inhibition is achieved.
2. Gamma interferon is liberated directly and dose-dependently. The release can be inhibited with dexamethasone and cyclosporin A.
3. The phytohaemagglutinin-induced release of gamma-interferon and interleukin-2 is inhibited in a dose-dependent manner.

Steps 1 and 2 of the hypothesis illustrated in Figure 9 are proved by the results of these tests. The results of these studies are represented in Figures 10, 11 and 12 (*Kalinski*, 1986, 1987; *Ottendorfer*, 1994; *Rosenkranz* and *Grundmann*, 1994; *Rusch* et al., 1986; *Rusch*, 1987).

In tests with mini-pigs, it was demonstrated that the oral administration of *Enterococcus faecalis* activates dormant B-cells leading to a prolifera-tion in the sense of a cascade reaction and eventually to an increase in the IgA synthesis (Figure 13). The stimulation of the activities of the mucosal immune system could be proved by the increase in secretory IgA's in the saliva of the mini-pigs (Figure 14). Steps 3 and 4 of the hypothesis illustrated in Figure 9 have been confirmed by the results of these tests (*Kalinski*, 1986, 1987; *Ottendorfer*, 1994; *Rosenkranz* and *Grundmann*, 1994; *Rusch* et al., 1986; *Rusch*, 1987).

Network

The complex mechanism of the mucosal immune system where the immune system, the nervous system, the hormone system and metabolism are interlinked is stimulated differently by different immunological signals of the microflora. Data from lymphocyte cultures of healthy volunteers can again serve as an example. In these tests live enterococci, live *E. coli*, a mixture of killed *Enterococcus faecalis* and *E. coli* (for oral administration) and killed *E. coli* (for parenteral administration) were studied. The live bacterial preparations were capable of promoting the production of interleukin-1ß; the liberation of gamma-interferon was not stimulated. On the other hand, the production of

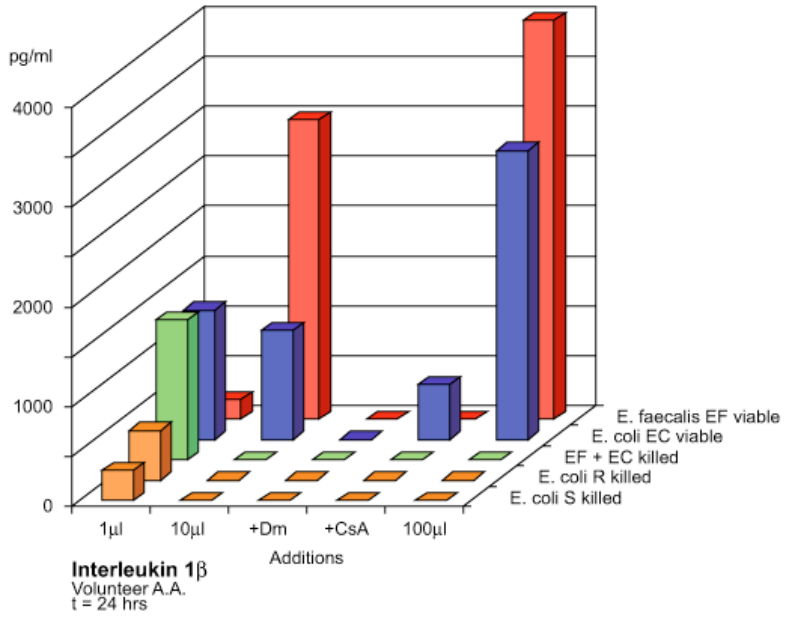

Figure 15: Influence of different bacterial preparations on the release of 1ß-interleukin in lymphocyte cultures of volunteers. Note the columns on the right (100 µl substance). The release is clearly promoted by live bacteria. The release is inhibited by the addition of dexamethasone (Dm) and cyclosporin A (CsA) (*Tarkkanen*, 1994).

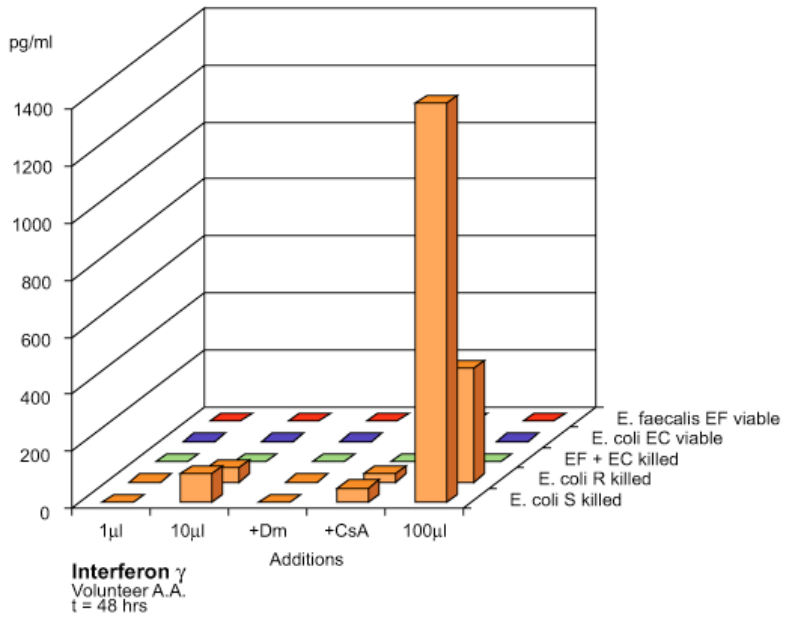

Figure 16: Liberation of gamma-interferon in lymphocyte cultures after stimulation with different bacterial preparations. The release of gamma-interferon is exclusively stimulated by killed *E. coli* and inhibited by the addition of dexamethasone (Dm) and cyxlosporin A (CsA) (*Tarkkanen*, 1994).

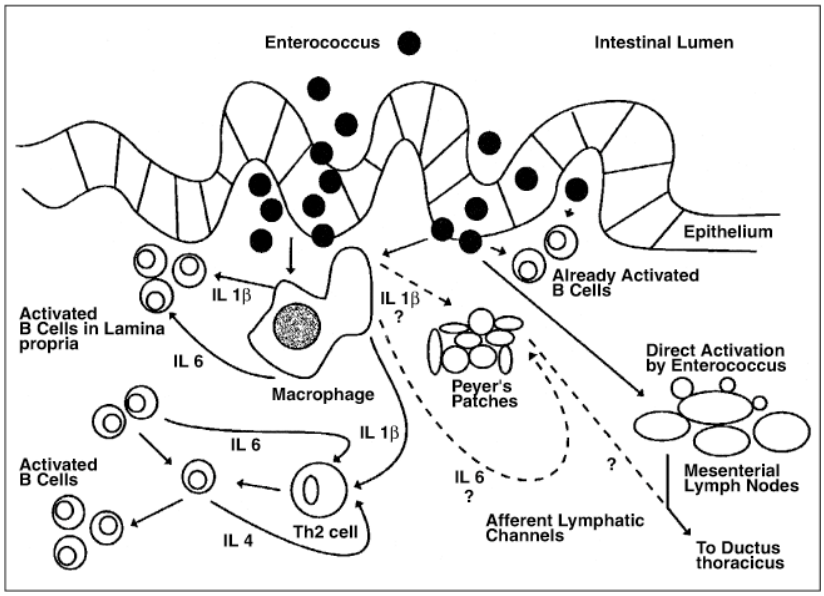

Figure 17: Schematic representation of the complex sequence in the stimulation of defence mechanisms of the mucosal immune system with *E. faecalis* demonstrated at the example of lymphatic tissue associated with the intestine (*Ottendorfer*, 1994).

gamma-interferon was stimulated by preparations of killed *E. coli* but not the release of interleukin-1ß. These data are set out in Figures 15 and 16. A further hypothesis, illustrated in Figure 17, is constructed on the basis of these data and the previously mentioned data illustrating the mode of action of microbial preparations based upon the example of the mucosal immune system associated with the intestine (*Jansen*, 1994; *Kalinski*, 1986, 1987; *Ottendorfer* 1994; *Panijel* and *Burkard*, 1993; *Rosenkranz* and *Grundmann*, 1994; *Rusch* et al., 1986; *Rusch*, 1987, 1989; *Schaffstein*, 1993; *Tarkkanen*, 1994).

Indications

Indications confirmed by controlled clinical trials are: immunomodulation (activation of endogenous resistance), colds, chronically recurrent infections of the respiratory tract, chronic sinusitis, inflammations in the mouth, nasal and pharyngeal cavity, middle ear, gastrointestinal disorders, irritable colon and urinary tract infections. Indications resulting from general experience are subject to controlled clinical trials: gastro-enteritis, enterocolitis, postantibiotic colitis, colitis ulcerosa, Crohn's disease, hepatopathies, cholecystopathies, skin diseases, allergic diseases and mycoses. All these indications concern areas of the human organism that are derived from the entoderm and cross-linked with the mucosal immune system. The wide spectrum of indications is explained by the mucosal immune system as a substrate (*Castro*, 1989; *Stern*, 1992). The inhibition of the phytohaem-agglutinin-stimulated clearance of gamma-interferon and interleukin-2 by *Enterococcus faecalis* in the *in vitro* lymphocyte cultures could become the pharmacological basis for further indications. The increased production of gamma-interferon and interleukin-2 under the action of the endotoxins of pathogenic Gram-negative bacteria could be reduced by *E. faecalis*. It is therefore feasible that *E. faecalis* could have some

importance in the treatment of hospitalism. This is indicated by the results of studies on lymphocyte cultures, animal experiments and human studies.

CONCLUSION

Microbial Therapy with microbial preparations and autovaccines is a therapeutic method based upon experimental and controlled clinical studies.

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OLD HERBORN UNIVERSITY SEMINAR ON PROBIOTICS: PROSPECTS OF USE IN OPPORTUNISTIC INFECTIONS

REVIEW OF THE INTERNAL DISCUSSION

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INTRODUCTION

Before probiotics can be discussed in a rational way, it is necessary to define the concept exactly. Furthermore, the mode(s) of action of different probiotics should be clarified and, finally, the potential fields of application of probiotics will have to be defined.

During the days for discussion the following topics were dealt with:

1. Definition of the concept
2. Enhancement of the colonisation resistance of the digestive tract.
3. Nutritional effects of probiotics, especially reduction of lactose intolerance.
4. Therapeutic effects of probiotics, with emphasis on carcinogenesis in the colon.
5. Biochemical functioning of different microbial ecosystems and the recognition of mechanisms which may allow flora control at this level.
6. Humoral and cellular immunomodulation of the host both at the mucosal and at the systemic level
7. Safety aspects of probiotics.

DEFINITION OF THE CONCEPT

Previous definitions

Several attempts have been made to define the concept of "probiosis". The following definitions have been proposed:

Parker (1974) : Organisms and substances which contribute to intestinal microbial balance.

Fuller (1992) : A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.

Havenaar (1992) : A mono- or mixed culture of live microorganisms which, when applied to animal or man, beneficially affects the host by improving the properties of the intestinal microbial balance.

Fuller (1994)*: A preparation of live microorganisms or microbial stimulants

* Proposed during the discussion.

which affects the indigenous microflora of the recipient animal, plant or food in a beneficial way.

Because of the paucity of information about the way in which probiotics work, the definitions already proposed are often too inexact to accurately define what is meant by the term probiotic. The discussion which followed sought to offer a better, more precise description of what is meant by probiosis.

Considerations leading to a new definition

A possible new definition obviously should not be biased by any of the shortcomings its predecessors suffered from. It should, therefore, not contain a statement concerning possible mode(s) of action and should not contain any ambiguities.

Although a possible new definition may be based on several different criteria, it should describe (i) the composition and (ii) the function of the probiotic. However, a definition of the desired function of a probiotic makes it quite difficult to distinguish the probiotic from food-additives (e.g. starter cultures) on the one side and genuine drugs (e.g. vaccines) on the other side. For this reason it was decided that the composition of a probiotic be regarded as the most important factor in the construction of a possible new definition.

Considering the composition, it was realised that a probiotic may exert different beneficial functions, both at the cellular and subcellular level. Examples of different probiotic functions at different levels were listed as: (i) living whole cells which are able to compete for substrate with potential pathogens and to produce antimicrobial substances against this latter group of bacteria (ii) dead whole cells which can contain enzymes like lactases (iii) peptidoglycan cell wall fragments which may be able to trigger a humoral or a cellular immune response. Therefore, because probiotic activity does not result from one substance or a defined mixture of substances, a possible definition should describe all components of a probiotic which are supposed to exert a beneficial function. General opinion was reached on: "A probiotic is a microbial preparation which contains live and/or dead bacteria including their components and products".*

The problem with this new definition is that it does not unambiguously discriminate between preparations like vaccines and starter cultures. It was suggested by Dr. Freter that the inclusion of a statement on the functioning of the probiotic should take care of the problem. A possible alternative then becomes: A probiotic is a microbial preparation which contains live and/or dead bacteria including their components and products, which is administered orally or to other mucosal surfaces, and which is intended to improve the microbial or enzymatic balance at mucosal surfaces or to stimulate specific or non-specific immune mechanisms". Because this suggestion was made after the meeting, there was no discussion on this alternative.

ENHANCEMENT OF COLONISATION RESISTANCE

During the third decade of this century Lotke and Volterra observed that an ecosystem is more stable when more niches are occupied. Consequently, the

* Dr. Fuller, Dr. Wallace, Dr. De Simone, Dr. Tannock, and Dr. Rowland do not accept this as a definition of probiotics.

more diverse an ecosystem, the more difficulty an invading organism will experience upon colonisation. However, it was not until 1956 that Freter and co-workers demonstrated that this mechanism was also applicable to the microbial ecosystem of the digestive tract, using pathogens like *Salmonella* spp. and *Shigella* spp. In 1971, van der Waaij and co-workers found that this rule extended to *potential* pathogens as well.

Before the active fraction of a microbiological preparation (i.e. the probiotic) can exert its function on the host it has to reach the digestive tract. Thereafter, it will have to stay biologically active in the digestive tract for some time. This latter property requires either adherence to the mucosal lining or a high division rate of the probiotic. Factors like "host-specificity" and "adaptation" may influence the colonisation of the probiotic to a considerable degree and were discussed separately. Finally, the function of the probiotic on the stability of the gut ecosystem and some specific examples will be treated.

Gastric acid kill

The acidity of the gastric environment amounts to about pH 2.0 under normal conditions. The main acidifier in gastric juice is hydrochloric acid, which is easily dissociated. Consequently, there is only a limited amount of organic material needed for strong buffering of the pH in the stomach. Selection of microbial strains for acid resistance is probably of minor importance, provided the probiotic is ingested with food or a storage medium with sufficient buffering capacity.

Neutralisation of the gastric pH by antacid drugs could facilitate gastric passage of a probiotic strain but has the disadvantage of allowing microbial overgrowth of the stomach. This phenomenon has two major disadvantages. Firstly, the increasing amount of (potentially pathogenic) bacteria in the stomach may result in a higher risk of translocation of the stomach flora to the gut and the lungs. Secondly, the increased number of bacteria in the stomach may antagonise the probiotic strain. During the discussion on this topic it was suggested by Dr. van der Waaij that antacid therapy (e.g. in gastric ulcer prophylaxis) could be accompanied by probiotic treatment. If a niche for the probiotic in the stomach ecosystem exists, the development of a potential pathogenic flora of the stomach could then be suppressed by the probiotic, while at the same time the probiotic will continuously be seeded from the stomach into the gut.

Adhesion and association

The microbial ecosystem of the lower part of the digestive tract can be divided in at least two subecosystems: the "luminal flora" and "mucosal flora". The composition of the "luminal flora" is mainly determined by the nutrients available and the effect of antimicrobial substances; the composition of the "mucosal flora" is mainly determined by the host's expression of specific adhesion sites in the enterocyte membrane, the mucus production rate, the production of secretory immunoglobulins and the extrusion of cellular material from the membrane into the mucus. Experimental data reveal that prolonged colonisation of the mucus ecosystem with a probiotic strain (after administration has ended) is difficult, if not impossible. For a newcomer it is only possible to remain in the digestive tract for a prolonged period when it is able to colonise the mucus blanket. Colonisation of the mucus blanket firstly requires the occupation of a ecological niche and secondly, multiplication with a division rate which is equal to, or higher than, the mucus-to-lumen transition rate. Occupation of a

niche in the mucus can be achieved by association with the mucosa. This may manifest itself either by cell-to-cell adhesion or by entrapment in the mucus. An adhering microorganism contains a ligand against which a receptor is present on the enterocyte membrane of the gut. This ligand can be made up of several macromolecular types (e.g. glycosaccharides or glycoproteins) and may have antigenic properties. In order to stay in the mucus it is necessary for the adhering microorganism to multiply. From the work of Freter and co-workers it has become clear, that an adhered microorganism has the selective advantage of a lower substrate turnover. In other words, the adhered microorganism does not need to multiply at high rate to remain present in the mucus. Consequently, because it can withstand mucosal wash-out while maintaining a slower metabolic rate, it can occupy niches which are too low on substrate for most other non-adhering microorganisms. Closer to the lumen, associated microorganisms withstand wash-out from the mucus by rapid multiplication without adherence. This group of microorganisms needs to have access to an excess of substrate and needs an efficient, probably oxidative, type of metabolism. The colonising ability of a probiotic strain depends, partly, on its ability to associate. Adherence may be tested *in vitro* but the results of these assays sometimes fail to predict the adhering (and thus colonising) properties of a bacterial strain. Reasons for this inconsistency could be (i) *in vitro* monolayers of enterocytes do not produce mucus (ii) enterocytes may *in vivo* express receptors other than those found *in vitro* and (iii) a monolayer does not produce secretory immunoglobulins. In the future there will probably be an increase in the use of human volunteers in research projects concerned with probiosis.

Host-specificity and adaptation

The current bibliography on the host-specificity of probiotic strains is limited. Apart from some anecdotal knowledge (e.g. *Lactobacillus* spp. have a higher host specificity than *Enterococcus* spp.) most hypotheses are based on indirect evidence obtained from experiments with germfree animals. Experiments with animals which have a very simplified gut microflora cannot give decisive results on the interaction between the host and its entire microflora on the one hand and with a probiotic strain on the other. Even when host-specificity for a certain probiotic-host combination can be demonstrated, it is still possible that the effect of the probiotic on the host differs from one host to another because interindividual differences in immunological history of the host may result in a different repertoire of secretory immunoglobulins in the mucus. At this moment it is not certain whether host-specificity does exist and, if it does, whether it results in a comparable effect in a wide range of different host animals.

There is more literature available on "adaptation" than on the issue of the "host-specificity". Experimental data on this topic comes mostly from gutflora transplantation experiments in decontaminated or germfree mice. A striking example of this phenomenon can be extracted from the work of van der Waaij and co-workers who inoculated decontaminated mice with samples of luminal flora from different species: chicken, cow, human, rat and sheep. After two weeks of incubation all mice had retained the flora they harboured before inoculation, except for the mice given the gut-flora of cows. The complex mixture of bacteria present in the lumen of the colorectal part for the bovine-gut had adapted to the intestinal environment of the mouse. Breeding with the individuals of this group of mice did re-

veal that the adaptation of a cow-flora to a mouse environment had detrimental effect on the offspring of these mice. All baby-mice died within three weeks showing symptoms like: growth retardation, severe diarrhoea, and severe loss of weight (Geertsma et al.: Microecology and Therapy 20, 447-452, 1990).

Probiotic influence on the gut ecosystem

The internal discussions on the influence of probiotics on the gut ecosystem concentrated on two intestinal disorders: diarrhoea and constipation. A third non-intestinal disorder, vaginosis, was also discussed.

Diarrhoea

At the moment many types of diarrhoea are known. They may differ with respect to progress (acute or chronic) or etiologic agent (virus, bacterium, parasite, fungus, microbial metabolites). Most of the research which has been performed in the field of probiosis and diarrhoea suffers from a black-box design. During the discussions some of the previously obtained results were mentioned:

1. 10^{10} cells of a lactobacillus starterculture per day can cure *Campylobacter cholerae*, *Yersinia enterocolitica*, *Salmonella* spp. or *Shigella* spp.-induced diarrhoea (Olukoya et al.: Journal of Tropical Pediatrics 40, 108-113, 1994).
2. *Bifidobacterium breve* 10^{10} cells per day can cure infantile *Campylobacter* enteritis (mentioned by Dr. Tanaka during the meeting).
3. There is little published evidence that Lactobacilli (i.e. *Lactobacillus* GG) can be used in the treatment of *Clostridium difficile*-induced diarrhoea (Gorbach et al.: Lancet ii, 1519, 1987).
4. *Saccharomyces boulardii* can be used in the treatment of *Clostridium difficile*-induced diarrhoea.

On the basis of the current experimental data no definite conclusions can be drawn. however, the following assumptions seem reasonable:

1. Probiotics will probably be more effective in preventing diarrhoea than in treating it.
2. Because of the self-limiting character of acute diarrhoea, probiotics are most likely to be employed in chronic diarrhoea.
3. The type of diarrhoea needs to be fully specified before probiotic treatment can be considered.
4. Because large differences exist between the effects that different bacterial species (even within the same genus) may exert on diarrhoea the probiotic needs to be fully identified.
5. In antibiotic-induced diarrhoea, it may be sensible to accompany treatment with antibiotics which have a high potential for inducing diarrhoea with a prophylactic probiotic treatment.
6. At this moment research in this field is limited by the absence of proper animal models for all the different types of diarrhoea.

Constipation

Two types of constipation are recognised: (i) constipation by blockade of the gut lumen (e.g. a tumour) while motility is still intact and (ii) decreased motility (especially in the elderly). Probiotic laxation is only rational in the second type of constipation. Traditional laxatives like bivalent salts or polyvalent alcohols have a non-specific mode of action. Therefore, pronged use of these substances may result in dehydration or disturbance of the electrolyte balance of the host. Though valid data on the underlying mechanism is absent, it may be possible that probiotic laxatives do not suffer from this side effect. This as-

sumption is supported by empirical studies of Dr. Tanaka and co-workers who found that oral administration yoghurts containing 10^{10} viable cells of *Bifidobacterium breve*, 10^{10} viable cells of *Bifidobacterium bifidum* or 10^{9} viable cells of *Lactobacillus acidophilus* per 100 ml to a group of bedridden patients on a low-fibre diet resulted in a significant increase of the bowel movement and a significant decrease of the constipation incidence (Tanaka: Japanese Journal of Geriatrics 19, 577-582, 1982). These beneficial effects were not accompanied by side-effects like dehydration or disturbance of the electrolyte balance.

Vaginosis

Production of hydrogen peroxide (H_2O_2) by lactobacilli seems to be an important factor in the maintenance of the ecological environment of the vagina. From the work of Hilier and coworkers (Obstetrics and Gynaecology 79, 369-373 (1992) it can be concluded that a stable population of H_2O_2-producing lactobacilli protects the vagina against bacterial invasion by pathogens of the female urogenital tract like *Gardnerella vaginalis*, *Candida albicans*, *Bacteroides* spp. *Peptostreptococcus* spp. and *Chlamydia* spp. The effect may even extend to viral pathogens like the human immunodeficiency virus type I (Seymour et al.: Journal of Experimental Medicine 174, 289-292, 1991). This protective function is of special importance when the normal vaginal ecosystem has been disturbed by e.g. pregnancy, antibiotic treatment or sexual intercourse. Local administration of H_2O_2-producing *Lactobacillus acidophilus* to women who suffered from bacterial vaginosis was found to be an effective therapeutic treatment (Hallen et al.: Sexually Transmitted Diseases 19, 146-148, 1992).

Lactobacillus acidophilus administered orally may also be employed in the prophylaxis of candidal vaginosis. (Hilton et al.: Annals of Internal Medicine 116, 353-357,1992). Curiously in this study the lactobacilli were ingested orally. However, the mechanism by which the lactobacilli enter the vaginal ecosystem remains unclear. These bacteria may enter the vagina after passage through the digestive tract. This seems plausible since the vaginal flora has many species in common with the rectal flora.

NUTRITIONAL EFFECTS OF PROBIOTICS

Several nutritional effects of probiotics have been investigated. In animal studies (McDonough: Journal of Food Science 47, 1463-1465, 1982) an increased growth rate has been observed after dietary supplementation with lactobacilli. Increased mineral absorption from the gut and the production of riboflavin and niacin, resulting from the temporary colonisation of the gut by lactobacilli, may partly explain the observation. However, the most convincing claims have been made in the probiotic treatment of lactose intolerance. Increased levels of intestinal lactase (i.e. ß-galactosidase) activity may help in the metabolism of lactose before bacterial enzymes can convert it to lactate. This latter substance may cause osmotic diarrhoea due to its relative slow absorption from the large intestine compared to the rapid absorption of short chain fatty acids, which, in turn, are able to enhance the absorption of water and electrolytes. In order to increase the levels of intestinal lactase activity, at least two strategies may be employed: (i) oral feeding of ß-galactosidase-producing

bacteria and (ii) oral feeding of a substrate specific for these strains. There are many studies in humans measuring the effect of lactobacillus fermented milks on lactose digestion. Some examples of these studies are (Gilliland and Kim: Journal of Dairy Sciences 67, 1-6, 1984; Hitchins and McDonough: Americam Journal of Clinical Nutrition 49, 675-684,1989) and, as part of a review on probiotics, (Gorbach: Annals of Medicine 22, 37-41 1994).

THERAPEUTIC EFFECTS

Colonic carcinogenesis

Epidemiological studies have shown that the incidence of colon cancer is higher among populations who have a low intake of fermented milks. One of the first reports of mutagenic activity in human faeces came from Bruce and coworkers (In: Hiat, H.H.; Ed.: Origins of human cancer, 1641-1646, 1977). Furthermore, it has been shown that the intestinal flora has the ability to metabolise endogenous and exogenous compounds (Bakke: Biochemical Pharmacology 30, 1641-1646, 1981). Usually conjugation by the liver leads to detoxification and inactivation of the compound. Deconjugation in the intestine by (bacterial) enzymes may regenerate the compound in the intestine. If the inactivated compound is a potential carcinogen then its reactivation in the intestine may enhance the risk of colon cancer of the host.

Biological markers

Measurement of the activity of some specified set of biological markers which are supposed to be indicative for the carcinogenic properties of a gut microflora may be useful in assessing the chance of the emergence of colon cancer. Biological markers fall in at least two functionally different categories: (i) the biological activity of mutagens in the intestines and (ii) the biological activity of (bacterial) enzymes which are supposed to catalyse the deconjugation of procarcinogens to carcinogens in the intestines. Biological markers of the first category include N-nitroso compounds, heterocyclic amines and secondary bile salts. Possible biological markers of the second category include azoreductases, nitroreductases, ß-glucuronidase and bile acid dehydroxylase. The activity of biological markers of the first category may be quantified by means of Ames' Salmonella/mammalian microsome test (Maron: Mutation Research 113, 173-215, 1983). However, these test methods do not give any information on the molecular composition of the mutagen(s) involved. The activity of biological markers of the second category can be quantified by measuring the speed at which specific substrates are metabolised by the gut microflora.

Effect on the host

Once the activity of a wide array of mutagens in the intestines is quantified, the next logical step is to assess the effect on the host organism. During the first day of the 8th Old Herborn University Seminar, Dr. Rowland presented an elegant method (the "Comet" assay) to measure the influence of potential genotoxins in the gut on the intestinal mucosal cells of the host. Briefly, a mucosal cell suspension is prepared from a biopsy of the gut epithelium and incubated with the substance or preparation to be tested for genotoxicity. The cells are then spread on a microscope slide in a layer of agar, lysed gently in alkali and subjected to electrophoresis. Any DNA damage

(strand breaks) induced by the test chemical causes DNA to "unwind" and spread out under the influence of the electric field to form a "comet tail" (visualised by staining with ethidium bromide). In general the length of the comet is proportional to the degree of DNA damage. The assay can be applied to *in vitro* and *in vivo* studies and cells isolated from human biopsies can be investigated. Clearly the relevance of such DNA damage to tumour initiation and promotion needs to be established. Ultimately, large scale human intervention trials on the influence of diet and/or probiotic treatment on the incidence of colonic cancer, and other types of cancer (e.g. breast cancer) will have to be performed. At this moment these types of studies have only been carried out on the influence of fibres on the colonic carcinogenesis.

Experimental data

During the discussions, the some experimental results were mentioned. Two of the most remarkable were:

1. A study of Aso and Akaza (Urology International (1992) 49; 125-129) in which the influence of oral feeding with *Lactobacillus casei* on the recurrence of superficial bladder cancer was investigated. In this study, oral feeding of the lactobacilli significantly reduced the recurrence of the tumour. However, because of its descriptive nature of this study, it is not clear what the molecular or cellular tumour suppressive mechanism is.
2. Daenen and co-workers (manuscript in preparation) found that rats with chronic leukaemia responded much better to chemotherapy when their gut microflora was modulated. In this study the facultative, anaerobic, Gram-negative rods (Enterobacteriaceae and Pseudomonadaceae) were removed from the flora. In the rats with the modulated flora, leukaemia developed less quickly and, because the response to the treatment was better, the mortality in this group was significantly reduced. The experiment, although no molecular mechanisms are known to date, clearly demonstrates that gut microflora plays an important role in carcinogenesis but also in its therapy.

Ammonia production

Although it was only mentioned briefly by Dr. Rowland, this aspect of host-gutflora interaction may become of more interest in the future because the recent developments in experimental and clinical hepatology have increased the survival rate of severely ill hepatological patients. As a result of this development the group of patients which are at risk of hepatic coma has also increased. If probiotic treatment can decrease the level of intestinal urease activity, the incidence of hepatic coma may be reduced considerably. Three possible mechanisms which may reduce the ammonia production of the gut microflora were proposed during the discussions: (i) substrate competition of the probiotic with the urease-producing inhabitants of the gut microflora (ii) probiotic production of antimicrobial substances which inhibit the growth of the urease producers in the gut and finally (iii) probiotic production of urea antagonists in order to reduce the intestinal urease activity.

BIOCHEMICAL FUNCTIONING OF THE GUT MICROFLORA

In contrast to the therapeutic effects, which were mentioned above, this section will deal with the biochemical functions of the gut microflora. With

this term the probiotic modulation of host-related characteristics is described. Until now there is one claim on which some scientific work has been performed i.e. the reduction of levels of serum cholesterol by means of probiotic microflora modulation. The potential clinical relevance of this category of probiotic treatment was stressed by Dr. van der Waaij who referred to the function of HDL in the transportation of endotoxin to the liver. Probiotic treatment may be applied in stimulating the clearance of endotoxin as long as liver functions are normal. The effect of probiotic supplementation is uncertain. Dr. Rusch mentioned some research that was performed at the Institute for Microecology in which the probiotic treatment of (mice) with *Escherichia coli* was without effects on the levels of serum cholesterol.

Even though the experimental data are inconsistent an attempt was made to suggest some mechanisms which may explain the phenomenon:

1. Utilisation of cholesterol by the probiotic. There is some *in vitro* evidence for this mechanism but because of the low efficiency of this process it is still not clear whether this phenomenon is not merely an artefact.
2. Formation of cholesterol inclusion bodies. During the discussions none of the participants was able to come up with references to studies concerning this subject.
3. Interruption of the enterohepatic cycle by elevation of the bile salt hydrolase activity in the gut microflora. This latter possibility cannot be regarded as beneficial because of the adverse effects of these enzymes on the membranes of the enterocytes in the gut epithelium.

Again, no decisive data on one or more of the effects mentioned above in humans is present today.

HUMORAL AND CELLULAR IMMUNOMODULATION OF THE HOST AT THE MUCOSAL AND AT THE SYSTEMIC LEVEL

Mucosal surfaces are continually exposed to antigenic material and the effectiveness of the ensuing mucosal immune response and the development of systemic non-responsiveness (tolerance) is of great importance in maintaining the health of the host. Probiotics may be useful in presenting extra antigenic information to the host thus enlarging its number of different T-memory clones. This extra immunological information may be of protective value when the host is faced with known, or related antigens, or when the host faces a large amount of known antigens. This principle of immunomodulation may become more important because of the growing number of immunocompromised patients and because of the growing number of pathogenic bacteria which are multiple resistant against antibiotics. Furthermore some of the functions related to the gut depend on the presence of non-pathogenic microflora which the immune system must learn to disregard.

Antigenic contact

Antigens present in the gut may come into contact with the Gut Associated Lymphoid Tissue (GALT) via 2 different pathways: (i) translocation over the enterocyte membrane and (ii) translocation over the membrane of M-cells which overlay the Peyer's Patches. In the first case, the antigen is most likely to be transported to the mesenteric lymph nodes by macrophages, while in

the second case the antigen may be presented locally to T- and B-cells by dendritic cells.

Macrophages.

The phagocytic, or non-specific immune system is comprised mainly of macrophages. The functioning of the phagocytic system can be assessed by measuring the amount of lysozymal enzymes released by macrophages or by measuring the amount of B- and T-cell stimulating factors like interleukins. Perdigon and co-workers (Infection and Immunity 53, 404-410, 1986) found that oral feeding of *Lactobacillus delbrueckii* spp. *bulgaricus* and/or *Streptococcus salivarius* spp. *thermophilus* (6 x 10^9 cells) resulted in a significant increase in the production of the lysozymal enzymes indicating an increased phagocytic activity. Bacteria were also administered via the intraperitoneal route but this route of administration would not be suitable for tests on human volunteers. The increased activity of the phagocytic system induced by lactobacilli administered orally was accompanied by a decreased translocation rate of enteric bacteria to lymphoid organs like spleen and liver (Perdigon: Journal of Food Protection 53, 404-410 (1990). However, these results were obtained in mice. In humans there are some experimental data (Rusch: Drug Research 44, 691-695,1994) but this author tested the activity of circulating mononuclear blood cells. Although no decisive experimental data were obtained in humans, the results of the studies on the non-specific phagocytic immune system may well account for the observation that probiotics stimulate a response to a wide range of non-related antigens.

Humoral immune system

Antigens originating from the gut which have passed the membrane of M-cells are phagocytised by macrophages. These cells synthesise and release interleukin-1ß and other cytokines. Interleukin-1ß is able to directly activate resting B-cells while it also triggers T-helper cells which subsequently start production of interleukin 2, which is essential for the proliferation of a resting B-cell to a mature plasma cell. Interleukin 6 has a function in the late stage of the B-cell proliferation. Once B-cells have developed their interleukin 6 receptor they differentiate to immunoglobulin producing plasma cells. Plasma cells in the GALT mainly produce secretory immunoglobulins, which are "sprayed" into the mucus. Antigens translocated to the spleen may induce a comparable response. However, in this case the antibodies produced are transferred to the circulation.

The cellular immune system

Besides B-cell regions there are T-cell regions in the Peyer's Patches. When a gutflora-associated antigen is translocated over the M-cell membrane, antigen presenting cells will induce T-helper cell generation resulting in both a humoral and a cellular immune response. Apart from the already mentioned induction of B-cell proliferation, T-helper cells also induce T-cytotoxic cells which, in co-operation with antigen presenting cells are responsible for the clearance of the antigen. Furthermore, T-helper cells will induce proliferation of T-memory clones. Immune complexes are formed early on during a primary contact of the (probiotic) antigen with the cellular immune system of the host These complexes stimulate T-helper cell generation necessary for the "switch mechanism" of immunoglobulin isotype from IgM to IgA antibodies in the GALT and for generation of B-memory. Subsequently, they generate the higher-affinity antibodies of the IgG isotype forming

immune complexes which neutralise and remove the antigen. The latter also activate the T-suppressor cell circuit which suppresses any further T-helper cell generation. Prolonged exposure of the host to a (probiotic) antigen may induce the T-suppressor clones to such a degree that the cascade of events leads to a decrease in both the humoral and the cellular immune reactivity but not in the phagocytic activity. This mechanism appears to be in concordance with experimental data in which a lowering of titres of circulating IgG against *Enterococcus faecalis* was observed after oral feeding of the strain to human volunteers (Jansen: Infection 21, 193-194, 1993).

In conclusion, it may be stated that probiotic immunomodulation will probably be one of the hardest problems to tackle because the system is antigenically complex (the gut-microflora) and is further complicated by the fact that individual hosts may have experienced profound differences in their immunological history, and therefore have large differences in the information stored in their T-memory clones.

SAFETY ASPECTS OF PROBIOTICS

The safety of a probiotic depends on several factors. The ones which were discussed most intensively during the 8th Old Herborn University Seminar were:

1. Ability to transfer genetic material.
2. Sensitivity to antibiotics.
3. Ability to produce hazardous substances.
4. Immune status of the host organism.
5. Non-infectious nature .

Regarding the first factor it was stated that the more competent a probiotic strain is to transfer genetic material (irrespective of whether the underlying mechanism is transduction, conjugation or transformation) the less it is suited for probiotic purposes. However, this problem may have a self-limiting nature because a large "plasmid-load" may have inhibitory effect on the multiplication rate of the cell. In other words: bacterial cells which harbour vast amounts of plasmids (and therefore have a high chance of carrying genetic information which may -when expressed- have a detrimental effect on the host) are less well suited to withstand the selective forces which maintain the ecosystem.

The second factor, although discussed separately, is merely an aspect of factor number one since resistance to antibiotics is often plasmid encoded. Although there are some reports of transfer of genetic material in the intestinal ecosystem, no convincing data concerning intestinal transfer of genetic information, which may be associated with pathogenity, is known presently. The third factor was not discussed intensively because it is obvious that probiotic strains should not produce any substance which may harm the host.

The fourth factor concerning the immunological handling of intestinal antigens by the host organism is more extensively dealt with in the previous paragraph. With regard to this issue it is sufficient to mention some of the most significant host-related factors which may determine the safety of the treatment: (i) the nutritional status of the host has a profound influence on the immunological defence capacity (ii) the immunological history of the host organism, in other words the antigenic information stored in the host's memory clones.

With respect to the fifth factor; probiotics need -of course- to be non-infective to both the normal and the immune impaired host.

Typical for the gaps in our knowledge of the host-gutflora interaction is the diversity in safety precautions which exists today. Some manufacturers of probiotics do perform their own research on the safety of their products (Rusch: Microecology and Therapy 10, 173-203, 1993) others do not. It is clear that much research on host-gutflora interaction needs to be done to reach a widely accepted set of safety regulations.