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

| 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. cricitus                     |
| B. eiksonii                    | 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. thermophilium               | L. lactis                      | S. intestinalis                 |
|                                | L. muralis                     | S. mitis                        |
|                                | L. plantarum                   | S. morbillorum                  |
|                                | L. reuteri                     | S. mutans                       |
| Enterococcus avium             | L. rogosae                     | S. oralis                       |
| E. faecalis                    | L. ruminis                     | S. ratus                        |
| E. faecium                     | L. salivarius                  | S. salivarius                   |
|                                |                                 | S. sanguis                      |
|                                |                                 | S. sobrinus                     |
|                                |                                 | S. uberis                       |

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.
DERIVATION OF LABORATORY ANIMAL SYSTEMS

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 β-glucuronidase activity in the caecal contents of RLF and RLFL mice. Male RLF mice had about 52% more caecal β-glucuronidase activity than did their female counterparts. Colonisation of male mice by lactobacilli reduced the β-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 oesophaga 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. *EcoRI* and *HindIII* 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 \cite{Rodtong, 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, \( \beta \)-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 \cite{Rowland, 1981}. \( \beta \)-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 \cite{Drasar, Barrow, 1985}. The major producers of \( \beta \)-glucuronidase in the large bowel are *E. coli* and obligately anaerobic bacteria such as *Bacteroides* and *Clostridium* species \cite{Drasar, 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 lactic acid bacteria 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 \cite{Brooker, Fuller, 1975; Barrow et al., 1980; Conway, 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 \cite{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 \cite{Mason, 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.

LITERATURE


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