Extended abstract

Microbial diversity in gut ecosystems

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Many animals of a wide range of orders have a portion of the digestive system adapted to accommodate a fermentation which aids them in digestion and provides them with a variety of nutritional and health benefits. The microbial community inhabiting the gastrointestinal tract is characterized by its high population density, wide diversity and complexity of interactions. The current knowledge of gut microbial diversity is almost exclusively based on the use of classical anaerobic culture techniques. Culture based studies have shown that all major groups of microbes - bacteria and protozoa [1,2], fungi [3], yeasts [4] and bacteriophages [5] - are represented in the gut. Importantly, it contains representatives of the three domains (Bacteria, Archaea and Eucarya) described by Woese and co-workers [6,7]. Populations have been described in herbivores, omnivores and carnivores and in all zoological classes ranging from insects to humans, and dinosaurs [1,2]. The human colon contains in excess of 10^{11} bacterial cells per gram of contents belonging to as many as 400 different species [8-10]. The rumen, the most extensively studied gut ecosystem, contains large numbers of bacteria (up to 10^{11} viable cells per ml, comprising 200 species), ciliate protozoa (10^{4} - 10^{6} per ml, spread over 25 genera), anaerobic rumen fungi (zoospore population densities of 10^{3} - 10^{5} per ml, divided into 4 genera) and bacteriophage particles (10^{9} -10^{9} per ml) [3,11-14]. Despite this vast amount of knowledge, culture-based enumeration and identification of community members have tremendous limitations.

The two major problems faced by gut microecologists are the inevitable bias introduced by culture techniques and the lack of a phylogenetically based classification scheme [15-19]. Modern molecular techniques based on sequence comparisons of nucleic acids can be used to provide molecular characterization while at the same time providing a classification scheme which predicts natural evolutionary relationships. In principle, nucleic acid probes can be designed to hybridize with a complementary target sequence and thus allow a complete description independent of the growth conditions and media used [6,15,20,21]. An example of the power of these modern molecular techniques is provided by the analysis of rRNA sequences.

The principles and practice involved in rRNA based methods have been extensively reviewed [6,17-19,21-26]. The highly conserved regions of the rRNA

molecules can serve as primer binding sites for in vitro amplification by PCR [27,28]. The more conserved regions are also useful, serving as targets for universal probes that react with all living organisms or domain probes discriminating between the broad phylogenetic domains Archaea, Bacteria and Eucarya. The more variable sequence regions are appropriate for the design of genus, species and sometimes even strain specific hybridization probes [26,29,30].

When rRNA targeted probes are designed in order to quantitate population abundance and activity in natural microbial communities, in which potential novel organisms reside, probe specificity is an essential consideration. Under appropriate hybridization and wash conditions, which must be determined experimentally, it is possible to discriminate between targets that differ by a single nucleotide [26]. Experimental evaluation of probe specificity needs to be preceded by rRNA database searches for target and non-target complementarity in order to identify mismatches. Probe design is a dynamic process requiring constant evaluation as more rRNA sequences become rapidly available. Probe specificity is further amenable to experimental testing, i.e. based on the "nesting" of probes. Since different degrees of conservation in rRNA sequences allow for design of general and specific oligonucleotide hybridization probes, a number of probes with increasing levels of specificity (e.g., family-, genus-, and species-specific probes) can be used to characterize a single micro-environment. If each set of probes covers the complete diversity of target species present, then the sum of amounts of 16S rRNAs quantified by a set of probes should equal the amount quantified by a more general probe. However, if the use of the general probe indicates that there is a significant amount of the target present in the sample not accounted for by the combined use of several specific probes then the presence of novel diversity and/or uncultured species and strains is suggested. This approach of probe nesting is well documented in the *Fibrobacter* studies of Stahl and coworkers [20,29,31] detailed below.

Oligonucleotide hybridization probes targeted towards 16S rRNA sequences provide a means for rapid and accurate measurements of population dynamics in mixed cultures [30,32,33]. The relatively high copy number of rRNA molecules per cell (10^3 - 10^5) makes it possible to detect individual fixed microbial cells with fluorescent end-labeled, rRNA-targeted oligonucleotide probes [15,34-37]. The major limitation of in situ hybridization lies in the need to adapt pre-hybridization cell permeabilization to accomodate different types of cell walls. This precludes the use of a single fixation technique for all members of a complex community. Membrane-based quantitative hybridization allows to measure abundance of a phylogenetically-defined microbial population in a sample by comparison of hybridization signal to signal intensities. It is possible to normalize concentrations of RNA samples with reference to a standard series and thereby quantitate relative abundance [2630,32,33,38]. Accurate characterization of microbial communities using membrane hybridization is dependent on an unbiased recovery of nucleic acids from environmental samples. Differential extraction and recovery of nucleic acid can result in immediate bias and needs to be considered in detail [19,21,26].

The use of rRNA-based methods in gastrointestinal microecology has become well established over the past five years. It was first demonstrated by Stahl et al [33]. Species specific 16S rRNA targeted

oligonucleotide probes were developed to enumerate strains of *Fibrobacter* (*Bacteroides*) *succinogenes* and *Lachnospira multiparus* in the bovine rumen to monitor shifts in population abundance or changes in population activity in complex microbial communities. Culture based enumeration of *F. succinogenes* was largely unsuccessful in the same study. These techniques formed the basis for further studies of bacterial populations in gut environments. Probes were developed for the other major ruminal cellulolytic bacteria *Ruminococcus albus* and *R. flavefaciens* and used to study the dynamics of bacterial interactions during fermentation of cellobiose, cellulose and alkaline hydrogen peroxide treated wheat straw [30,32]. The results provided useful information on bacterial competition during growth on insoluble substrates. Furthermore, this study revealed for the first time the production of a bacteriocin-like substance by a ruminal bacterium, a mechanism which may be used to compete for nutrients. The ruminal bacterium *Synergistes jonesii* has attracted considerable interest due to its limited geographical distribution and its ability to degrade 3,4-DHP, the toxic principle of the tropical leguminous shrub *Leucaena leucocephala* [39,40]. The 16S rRNA sequence of *S. jonesii* was not closely related to any among those bacteria so far characterized and was an ideal candidate to evaluate the use of oligonucleotide probes for tracking bacteria in the ruminal ecosystem. Radiolabeled and fluorescent-dye-conjugated probes were developed for quantitation of *S. jonesii* in a mixed-culture chemostat [38]. Further ecological studies on transmission, colonization, persistence and population studies in vivo are under investigation.

In the human colon, probes specific for the *B. fragilis* ensemble, which represent over 30% of the normal culturable human faecal flora, have been developed by Doré and co-workers [41]. Colony hybridization was used to enumerate total *Bacteroides* after growth on a non-selective medium in conjunction with a *B.vulgatus* monoclonal antibody in order to examine populations in different age groups. Langendijk et al [36] developed a genus specific probe for *Bifidobacterium* spp. in human faecal samples. Cells were detected using fluorescein isothiocyanate-labeled probes and enumerated by an image analysis system which allows fluorometry of individual cells. Their results based on 16S rRNA hybridization and classical cultivation techniques indicated that almost all bifidobacteria were culturable. However, since the total culturable contents were only a fraction of the total microscopic counts, the contribution of bifidobacteria to the total intestinal microbiota was overestimated by almost 10-fold when cultural methods were used as the sole method for enumeration. Kok et al [42] similarly enumerated a probiotic *Bifidobacterium*.

Another promising application of in situ hybridization techniques is the topological localization of microorganisms. For instance, localization on mucosal or cell surfaces is usually performed by classical histological and immunohistological techniques, which restrict identity of the bacteria involved to morphological features. Even when specific antibodies are available for in situ studies, the thick mucus layer above epithelial cells can block penetration of antibodies and extensive washing can remove the mucus layer displacing organisms. Poulsen et al [43] applied in situ 16S rRNA hybridization to

investigate the microbiota of the large intestine and were able to determine the spatial distribution of *E. coli* in thin sections of intestinal tissue. This allows rapid detection of bacteria which may be difficult to cultivate and the investigation of their relationship to other cells either host or bacterial. They also determined in situ growth rates showing that adherent microorganisms were growing with generation times of 30-80 min while those in the lumen were static [44].

The hydrogen-utilizing anaerobes represent a case-study of their own, and considering the limited number of isolates obtained to date, the appreciation of their diversity is likely far from complete. The lack of isolates is partly the result of technical difficulties with cultivation. Doré and co-workers [45] have developed a quantitative hybridization technique using total RNA extracted from frozen faecal samples to study the distribution and population levels of *Methanobrevibacter* spp. The detection limit for the method was 10^6 methanogens per gram faeces. Above this level, hybridization and classical cultivation correlated well with one exception suggesting the presence of a non-cultivable or unique methanogenic group. Studies of this nature which target further population groups (dissimilatory sulfate reducers and acetogens) will provide important insight into this variable and controversial topic.

Surprisingly, a limited number of studies have used rRNA based methods to study ruminal protozoal and fungal populations. Comparative sequence analysis of the 18S rRNA genes was used to phylogenetically position *Neocallimastix* in the Chytridiomycete class of fungi [46]. This and further partial sequences have been used to design a total-fungi probe and a set of genus-specific probes (Doré et al., unpublished). These probes have been used in conjunction with probes targeting cellulolytic bacteria to investigate rumen cellulolytics in different farm and wild ruminants (Fonty et al, personal communication). Finlay et al [47] used a fluorescently labeled oligonucleotide probe targeting the 16S rRNA of the Archaea to demonstrate that *Entodinium* species and *Dasytricha ruminantium* contained methanogenic endosymbionts outside digestive vacuoles. Exosymbiotic methanogens had been well documented previously based on characteristic autofluorescence of these archaea [48].

Future prospects will include the direct assessment of molecular diversity as is now possible using different PCR-based rRNA-targeted methods. Comparative sequencing of rDNA genes cloned from environmental DNA extracts or the electrophoretic analysis of PCR-amplified rDNAs under denaturing conditions (DGGE, TGGE) are among the approaches currently applicable to the gut environment. Indeed, gut microecologists have not systematically examined this aspect which has great significance, considering the fraction of the microflora that eludes cultivation. Wilson et al [49] gave the first results of a direct study, applied to the faeces of a human individual.

In providing specific and sensitive, culture independent, evaluations of all members of the gastrointestinal ecosystem, new molecular technologies will revolutionize our appreciation of the diversity of the gastrointestinal microflora and allow a potentially complete description of the ecosystem [24,50]. Rather than replacing the classical culture-based system, the new molecular techniques should be used in combination with the

classical approach in order to improve cultivation, speciation and evaluation of biodiversity. In that respect, it is worth noting that the comparative rRNA sequence database is largely the result of pure culture studies. Finally, the combined application of population and activity (gene) probes will enable microbial ecologists to determine the exact role a specific organism plays in the natural environment and its quantitative contribution to the whole.

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