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# Methods for Analysis of the Intestinal Microflora

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## Abstract

**The concept of probiotics has been around for about 100 years. Yet its impact on human nutrition is still an emerging concept. Lack of convincing scientific validation for the efficacy of any ingested probiotic bacterium on intestinal health, has been a major reason for the low impact of probiotics on human nutrition. Obtaining positive scientific validation requires the use of suitable probiotic strains and also the necessary tools to monitor the performance of these bacteria in the intestines of individuals. To date, selection of strains for probiotic purposes has not been based on a scientific directed approach, primarily because it is not yet fully known what specific traits a desirable probiotic strain should possess. Filling this knowledge void will depend largely on furthering our understanding of the human intestinal ecosystem and the functional role of specific bacteria for intestinal health. Traditional approaches for studying this ecosystem have provided a good foundation in this knowledge base. Complementation of the traditional approaches with the emergence of sophisticated molecular tools shows enormous promise for obtaining the necessary insight into the intestinal microflora. This review will cover the traditional methodologies which have been used to analyze the human intestinal microflora. It will also reveal the development of modern molecular approaches for studying the diversity and phylogeny of its flora, and the rapid molecular tools for monitoring the presence of specific strains in the intestine. Finally, it will address the advent of *in situ* analysis of individual microbial cells, which promises to provide tremendous advances in our understanding of the microflora and their metabolic activities in the human intestine.**

## Introduction

Although tremendous strides have been made, our knowledge of the ecology of the human intestinal microflora is largely still in its infancy. This natural ecosystem represents one of the most complex and concentrated group of microorganisms in nature. Adding to the complexity is that each individual's intestinal ecosystem may have its own distinct characteristics and those characteristics are not uniform over time. Understanding the diversity and role of individual microbes in the intestine has been hampered by the lack of adequate methodologies. The recent advent of molecular methodologies has greatly aided the analysis

of the intestinal microflora. Future refinement and expansion of these molecular approaches will potentially unveil intricate details of this unique ecological niche. This review will outline the classical approaches for examining intestinal microflora and the recent evolution of molecular approaches, which have greatly complemented them.

## Culture Techniques for Intestinal Microflora

To date, essentially all our knowledge of the intestinal microflora has been obtained from isolating organisms from faecal or intestinal material by culturing and subsequently analysing them. This approach is still the mainstay for studies on the human intestinal ecosystem. However, cultivation of microbes as a means to characterise microbial communities in a natural ecosystem has major shortcomings, as it is recognized that many microbes in different ecosystems cannot be cultivated by standard culture techniques (Ward *et al.*, 1990). Despite the limitations, culture techniques are very powerful and absolutely essential to obtaining a complete picture of the diversity and role of the intestinal microbial ecosystem. To study such a complex ecosystem, the combination of both culture and molecular based non-culture techniques are required (Palleroni, 1997).

## Non-Selective Culture Methods

The essence of culturing techniques involves plating out fresh faecal or intestinal material on either selective or non selective media and incubating at 37°C. Samples are generally homogenized in a sterile liquid, such as 0.1% peptone water. Non selective media are generally used to estimate total numbers of both aerobic and anaerobic flora. Examples of non selective media that have been used for this purpose are rumen fluid-glucose-cellobiose agar (RGCA) (Moore and Holdeman 1974); modified medium 10 (Wilson and Blitchington, 1996); plate count agar (Alander *et al.*, 1997); brucella blood agar (BBA) supplemented with 0.5% sheep blood, 1 mg/ml vitamin K<sub>1</sub> and 5mg/ml hemin (Langendijk *et al.*, 1995); and brain heart infusion (BHI) (Ramare *et al.*, 1996). It should be emphasized that while these media contain no known selectivity power, they do inherently select against some bacteria from the human intestine. Specifically, bacteria that require extra requirements and also microbes that could grow on the media but may be in a physiological state which may not be conducive to culturing directly from faeces or intestinal material.

## Selective Culture Methods

Enumeration of specific bacterial genera is generally achieved by plating on selective media. *Bacteroides* species are the most numerically dominant bacteria and therefore can be isolated without selective agents added to the media. However, several selective agents such as bile, esculin or antibiotics can be used for selective enrichment for *Bacteroides* species (Engelkirk *et al.*, 1992).

The use of these selective agents is thought to inhibit many colonic strains and would therefore underestimate the *Bacteroides* count. Plating on non selective media and subsequently identifying *Bacteroides* can give a more accurate assessment of the numbers of this genera present (Corthier *et al.*, 1996). *Bifidobacterium* is another dominant genus found in the human intestine and a number of selective media for their enumeration have been developed. Common bifidobacteria selective media that have been used for analysis of the human intestine are, YN-6 (Resnick and Levin, 1981); Pentuey's selective medium (PSM) containing pyruvic acid and naladixic acid (Tanaka and Mutai, 1980); BS1 (Mitsuoka *et al.*, 1965); BIM-25 (Muñoz and Pares, 1988); and Beerens medium (Beerens, 1991). Bifidobacteria selective agents in these media mainly include antibiotics (kanamycin, naladixic acid, paramycin and polymyxin B) and/or propionic acid. However, analysis of some commonly used bifidobacteria selective media found that none were fully selective and that they generally contained toxicity against some bifidobacteria (Silvi *et al.*, 1996). Other intestinal bacteria such as lactobacilli, which are major inhabitants of the small intestine, are commonly cultured from faecal or intestinal samples using either Rogosa (Difco) or acidified Man Rogosa Sharpe (MRS; Difco) or LAMVAB (Hartemink *et al.*, 1997) media; clostridia, which are present particularly in older individuals, can be isolated from human faeces using novobiocin colistin agar (NCA) and colistin crystal violet agar (CCA) (Fujisawa *et al.*, 1995); enterococci and fecal streptococci can be isolated using Stanetz-Bartley (SB) medium, also called Bacto m Enterococcus Agar (Difco), or oxolinic acid-esculin-azide (OAA) (Audicana *et al.*, 1995); and *Enterobacteriaceae* can be isolated using MacConkey agar (Difco). All these selective media are valuable tools for analysis of the ecology of the intestinal microflora. However, they all have the inherent disadvantages of not absolute selectivity and toxicity against certain strains within the genus. In addition, all culture media fail to cultivate organisms which are in a physiological state which is not conducive to growth, often termed a 'non-culturable' state.

### Classical Approaches for Characterising Intestinal Microflora

Classical techniques for analysing intestinal microflora include both culture-dependent and culture-independent approaches. Both strategies have contributed in significant ways, but are inherently limited by lack of precision and are labour intensive, thus limiting their effectiveness for analysing a large number of individuals.

#### Classical Culture-Dependent Techniques

Culture techniques as outlined above are used to isolate culturable bacteria from faecal or intestinal samples. Upon isolation of colonies it is then necessary to confirm the genus identity and also further characterise to the species (or strain) level. This characterisation requires a battery of classical morphological and biochemical tests, many of which can be obtained from the Bergy's Manual of Systematic Bacteriology (Bergey, 1986). The confidence level of the species identification will increase with the more

tests that are carried out. Therein lies the greatest disadvantage of classical tools for identification of organisms, as even the most sophisticated array of tests can often lead to uncertainties in the classification of isolates. These tools are also ineffective in comparing the relatedness between species from different individuals. This is an important point as the future of probiotics depends on being able to predict how suitable a particular strain would be to the intestines of different individuals. For this level of characterisation of evolutionary relatedness, tools more precise than morphological and biochemical are needed.

#### Classical Culture-Independent Techniques

Total reliance on culturing for analysing the microflora of an ecological niche would give a very uncertain picture, as there would be no way of knowing how effective the culturing methods were for the bulk of the organisms present. Fortunately, a number of classical tools have been able to give valuable insight into the real numbers of microflora *in situ* in faecal samples. However, these tools are very limited in their ability to give any in depth characterisation of specific organisms. These techniques include direct microscopic analysis and monitoring specific enzymes or metabolites.

##### Direct Microscopic Analysis

The light microscope has been a valuable tool for estimating the total number of bacteria in faecal samples. Approximately  $10^{11}$ -  $10^{12}$  organisms per gram of wet faeces have been reported (Langendijk *et al.*, 1995; Holdeman *et al.*, 1976). However, the microscopic technique itself is not infallible and may significantly under report the true numbers. The technique generally used involves heat fixation and staining (Holdeman *et al.*, 1977) and detachment of cells is likely to occur, especially during washing. Furthermore, not all cells are conducive to the stains used. Despite limitations, direct microscopic analysis does give a good indication of the total microbial population numbers present in faeces and this is a valuable aid for assessing how effective a culture methodology may be for analysing the intestinal microflora.

##### Enzyme/Metabolite Analysis

Measurement of specific enzymes and metabolites in faecal samples can indirectly give information on the presence of specific microflora, or to be more precise, on the metabolic activities of specific groups of microflora. This indirect approach can be quite rapid and therefore, can allow the analysis of a large number of individuals. It is also advantageous as it gives important functional information on the metabolic activities of the bacterial microflora. Short chain fatty acids (SCFA), of which the principle ones are acetate, propionate and butyrate, are end products of anaerobic bacterial fermentation. Measurement of these acids in faeces can be correlated with specific bacterial metabolism in the intestine (Rowland, 1989). Increases in SCFA, which is considered a desirable trait, can point to increases in metabolic activities of primarily lactic acid bacteria. For example, *Lactobacillus casei* GG fed to children with an intestinal infection significantly increased the total SCFA concentration (Siigur *et al.*, 1996). Increases

in specific SCFA can point to increases in the metabolic activity of specific genera. For example, supplementing the subjects diet with bifidobacteria was found to result in a significant increase in acetate production (Jiang and Savaiano, 1997).

Increases or decreases in specific enzymes in faeces can also point to the metabolic activities of certain groups of bacteria. For example,  $\beta$ -glucuronidase, which has been implicated in colon carcinogenesis (Goldin and Gorbach, 1984), was shown to be significantly reduced in humans during ingestion of *L. casei* GG (Ling *et al.*, 1994). Also, a significant correlation has been implicated between the levels of faecal  $\beta$ -galactosidase and numbers of bifidobacteria (Favier *et al.*, 1997). However, at present, it is generally not feasible to accurately correlate many faecal enzymes with the presence of a specific microflora. Many faecal enzymes, such as, azoreductase and nitroreductase can generate toxic metabolites in the intestine (Rowland, 1989). While species of *Bacteroides*, *Eubacterium* and *Clostridium* are likely candidates responsible for these two enzymes, more studies are needed to accurately correlate specific faecal enzymes with specific groups of bacteria. Recently Wolin *et al.*, (1998) developed a detection method for  $^{13}\text{CH}_3^{13}\text{COOH}$  from 3- $^{13}\text{C}$ -Glucose, which is a characteristic end product from bifidobacterial glucose fermentation, in the faecal suspensions of infants. This potentially may be a very useful and accurate indicator of

bifidobacterial metabolism in the intestine. Further studies are needed to identify signature metabolites for other intestinal microflora.

### Molecular Advances for Typing and Phylogenetical Characterisation of the Intestinal Microflora

Classical culture techniques for the isolation of microbes from the human intestine is the sole source of intestinal microflora. Identification and characterisation of the resulting isolates by classical methods has many shortcomings, in particular, lack of accuracy and it is labour-intensive. The advent of molecular tools has greatly expanded the ability to reliably identify isolates and also to calculate the evolutionary relatedness between strains. Fingerprinting techniques (discussed in the next section), primarily DNA based, can be used for identification, but this strategy is limited by the extensiveness of the particular fingerprinting database. As databases for the different fingerprinting techniques grow, this approach will increase in usefulness. A major advantage of using a fingerprinting approach for typing purposes is its rapidity and, consequently it is conducive to analysing a large throughput of unknown isolates. A disadvantage, can be the sensitivity of the particular fingerprinting technique. While the sensitivity of the different fingerprinting techniques varies quite a bit, many common techniques do not have the

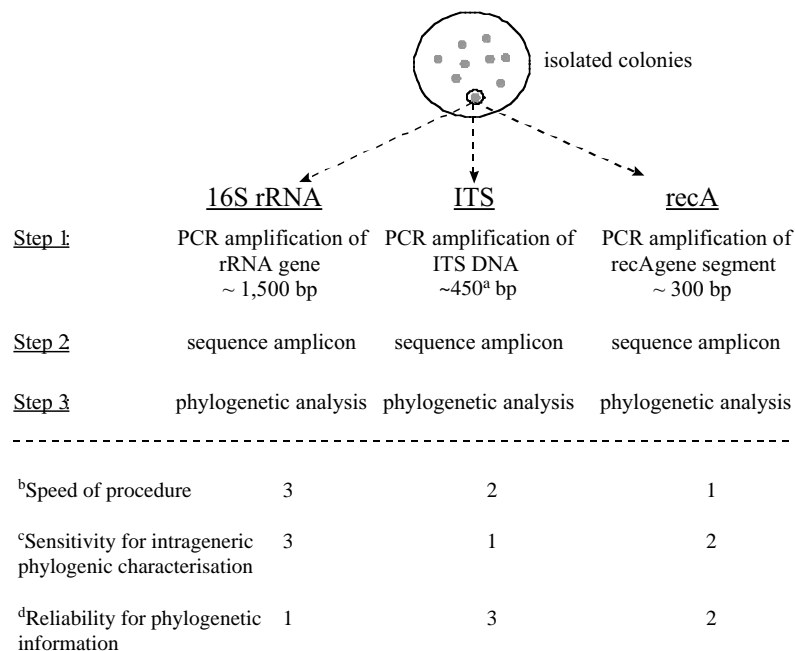


Figure 1. Comparison of the three sequence-based typing and phylogenetic characterisation approaches which have been used to characterise human intestinal isolates. The three sequences which have been used are 16S rRNA; sequence between the 16S and 23S rRNA genes, termed the internal transcribed spacer (ITS); and an internal portion of the *recA* gene. In each case the sequences were obtained via PCR using primers which are directed to universally conserved target sequences in each instance. <sup>a</sup>, represents the average ITS size of 29 bifidobacteria isolates, calculated from Leblond-Bourget *et al.*, (31); <sup>b</sup>, numbers 1 - 3 represent rankings, with '1' representing the fastest procedure; <sup>c</sup>, compares how sensitive the procedures are at differentiating isolates, with '1' representing the most sensitive; <sup>d</sup>, represents theoretical and experimental evidence for the reliability of each molecule at evaluating phylogenetic relationships, with '1' indicating the most reliable. Note, the 16S rRNA molecule is the only one capable of evaluating phylogenetic relationships between multiple genera. Both the ITS and *recA* only have value for intragenetic characterisation.

sensitivity to differentiate between strains and in some cases, between closely related species. These low sensitivity fingerprinting techniques also have a limited ability to discern the phylogenetic relationship between isolates. These disadvantages limit the effectiveness of many fingerprinting techniques for accurate typing of unknown isolates and evaluating their phylogenetic relationships. It is however, an extremely powerful tool for monitoring known bacterial strains and is therefore the tool of choice for tracking the prevalence of certain intestinal isolates within a population.

### 16S rRNA Sequence Analysis

Accurate typing of unknown isolates is now achieved by sequence analysis of 16S ribosomal RNA (rRNA). This tool for classifying organisms and evaluating their evolutionary relatedness was first developed by Woese and coworkers (Woese, 1987). The available database of rRNA sequences is now extensive, which allows detailed studies to be made on the phylogenetic position of unknown isolates. This molecular phylogeny approach has revolutionized the field of microbial ecology and has allowed meaningful phylogenetic relationships between microbes in natural ecosystems to be discerned (Olsen *et al.*, 1994). Technically, this is very feasible as the polymerase chain reaction (PCR) can be used to directly amplify the 16S rRNA gene directly from colonies using primers which are directed at universally conserved regions at both ends of the gene. The entire PCR amplicon, which is ~ 1.5 kb can then be directly sequenced and compared to the rRNA database (Figure 1).

This technology has greatly helped our understanding of the phylogenetic relationships between the major genera in the human intestine. The two major genera, *Bacteroides* and *Bifidobacterium*, are very heterogeneous and the use of 16S rRNA sequence analysis has contributed enormously to their phylogeny (Leblond-Bourget *et al.*, 1996; Shah and Collins, 1989). Understanding the phylogeny of bifidobacteria is particularly important, as members of this genus are prime candidates for inclusion in probiotic cultures for human consumption. Without comparative studies on the dominant bifidobacteria present in the human intestine, there is limited scientific rationale for selecting specific strains for probiotic purposes.

### ITS Sequence Analysis

Within the genus *Bifidobacterium*, the rRNA sequence is highly conserved (Leblond-Bourget *et al.*, 1996) and may not be sensitive enough for the desired level of comparative analysis that is likely to be needed for selection of worthwhile strains. Ideally, extensive phenotypic analysis would complement this approach and provide the level of analysis needed for rational strain selection. Indeed, this combined approach is thought to be the most powerful approach for understanding the true phylogeny of microbes and is emphasized in a recent review (Palleroni, 1997). However, this strategy is too labour intensive for high throughput of organisms from the intestinal ecosystem. To complement the rRNA sequence approach, analysis of another molecule, which is not as conserved as 16S RNA but still retains the characteristics of a meaningful phylogenetic marker, is required. Two important criteria for

such a molecule are, that it is universally present in bacteria and it has high sequence conservation, which illustrates that sequence changes are less influenced by temporary environmental changes. The region between the 16S and 23S rRNA genes, termed the internal transcribed spacer (ITS), has been used for a more detailed analysis of bifidobacteria (Leblond-Bourget *et al.*, 1996). This molecule is universally present in bacteria, but can exhibit very low sequence conservation (Barry *et al.*, 1991), thus limiting its accuracy as a phylogenetic marker. In addition the ITS regions within the same bacterial strain can exhibit heterogeneity (Christensen *et al.*, 2000; Garcia-Martinez *et al.*, 1996). However, the molecule is technically very feasible to obtain as PCR can be used to amplify the molecule directly from colonies using primers directed at universally conserved regions within the bordering 16S and 23S rRNA genes (Figure 1). Leblond-Bourget *et al.*, (1996) did evaluate the sequence analysis of this molecule for further characterising bifidobacteria and did find it gave much more sensitivity than the rRNA analysis. More recently, Tannock *et al.* (1999) demonstrated its usefulness for identification of intestinal *Lactobacillus* spp.

### recA Gene Sequence Analysis

Recently, a short segment of the *recA* gene has emerged as a potential candidate for a sensitive molecule for determining intragenetic phylogenetic relationships and is amenable to large scale analysis of a natural ecosystem, such as the human intestine. It possesses the important criteria of being universally present in bacteria and also being highly conserved. The *recA* gene encodes the RecA protein, which plays vital roles in recombination, DNA repair and the SOS response (Roca and Cox, 1997). Studies have established that meaningful bacterial phylogenetic relationships can be obtained by sequence analysis of the RecA protein (Eisen, 1995; Karlin *et al.*, 1995). These studies highlighted the possibility that a segment of the *recA* gene might be a useful molecule for phylogenetic analysis within a particular genus. This concept was applied to the genus *Bifidobacterium* in a recent study by Kullen *et al.*, (1997a). The molecule was obtained from both type and intestinal bifidobacterial isolates direct from colony isolates using PCR with primers directed to regions within the *recA* gene, which are universally conserved in bacteria. This approach yielded a fragment of ~ 300 kb, which could be rapidly sequenced using a single sequence reaction from either end (Figure 1). The phylogenetic relationship obtained by sequence analysis of this short segment of the *recA* gene, compared favorably with the analysis from the complete rRNA gene. Given the rapidity of obtaining the sequence information of this *recA* molecule, coupled with its theoretical and experimentally substantiated role as a meaningful phylogenetic molecule, it is potentially a very valuable tool for comparative phylogenetic analysis of human intestinal bifidobacterial isolates.

### Modern Approaches for Monitoring the Distribution and Prevalence of Specific Microbes in the Intestine

Rapid analysis of colony isolates is an important feature of any approach to study the intestinal microflora on a large scale by culturing methodology. Fortunately, a number of

rapid detection or fingerprinting approaches have been developed for this purpose, which can provide valuable information on the range of different types of isolates. Many of these rapid techniques also provide information on how related different strains are to one another. While this comparative analysis is generally less sensitive than the sequence based methods discussed in the previous section, it is a valuable first step to divide the isolates into broader groups, prior to the more elaborate sequence based methods.

### Phenotypic Fingerprint Analysis

Fingerprint techniques have been developed which are directed at both phenotypic and genotypic characteristics. Although phenotypic fingerprints can be obtained, these are usually less sensitive and changes in the fingerprint may not necessarily mean a different organism, but rather could be due to a change in expression of the particular phenotypic trait. Examples of phenotypic fingerprints are polyacrylamide gel electrophoresis of soluble proteins, fatty acid analysis, bacteriophage typing and serotyping. The most rapid and useful of these procedures is serotyping, as colonies can be directly typed, without sub-culturing, by colony hybridization with a monoclonal antibody specific for a particular genus, species or strain. This strategy has been applied for the analysis of two *Bacteroides* species in different human intestines (Corthier *et al.*, 1996).

### Genotypic Fingerprint Analysis

The recent development of multiple genotypic fingerprinting methodologies has been a major advantage for deciphering the complex human intestinal ecosystem. While all possess limitations, each can contribute to our understanding of the diversity of the different types of dominant microbes present in the intestine of different individuals. The first molecular detection system developed was hybridization with a nucleic acid probe targeted at a specific DNA sequence. While this is an elementary fingerprinting technique, more sophisticated methodologies have since been developed. Those which are potentially useful for the study of the human intestinal microflora are discussed below and a comparison of these fingerprinting techniques is summarized in Table 1.

### Colony Hybridization with Nucleic Acid Probes

A nucleic acid probe is a labelled single-stranded nucleic acid that can specifically hybridize (bind) to its complementary sequence. Probes therefore can target specific sequences in a genome. These target sequences are generally chosen such that they are unique to the particular genus, species or strain. Technically, the procedure is rapid as colonies can be directly probed, by lysing the colony to expose the nucleic acid content and allowing access for the probe. The label on the probe can be either radioactive, enzymatic or fluorescent, which can be readily detected. The selection of probes however, is the key to success with this approach, as any cross reactivity can give ambiguous results. Probes can be obtained using either a shotgun or a directed approach. The shotgun approach is to randomly isolate DNA fragments and test them for probe reactivity against a bank of isolated strains. This approach has been used to obtain strain- and species-specific probes for bifidobacteria (Ito *et al.*, 1992; Mangin *et al.*, 1995) and a species specific-probe for *Bacteroides vulgatus* (Kuritza and Salyers, 1985). However, these probes have yet to be tested in large scale studies of human intestinal isolates.

The directed approach to probe selection leaves less to chance, as it relies on choosing probes directed at target sequences which are thought to be unique to the particular microbe or group of microbes under study. One strategy is to identify enzymes unique to a group of organisms and direct probes at targets within the enzyme gene sequence. A potential example would be the bifidobacterial enzyme, fructose-6-phosphate phosphoketolase (F6PPK), which is used by members of this genus to metabolise carbohydrates via a unique pathway often called the fructose-6-phosphate shunt. As this enzyme is unique to this genus (with the possible exception of *Gardnerella*), it potentially is a good source of probes for this genus and should afford an effective means for tracking these bacteria in the gastrointestinal tract. However, the sequence of this gene is not yet available.

Generating short (~ 20 bases) oligonucleotide probes directed at regions of the rRNA is the most common means of obtaining genus- and species-specific probes. This approach is possible as there are some short variable sequence regions within this molecule, which can

Table 1. Comparison of Some Useful Molecular Fingerprinting Techniques

	PFGE	Ribotyping	16S rRNA RFLP	Multiplex PCR	AP-PCR (RAPD)	TAP-PCR
Requires culturing	yes	yes	no	no	no	no
<sup>1</sup> Rapid technique	no	no	yes	yes	yes	yes
Discriminatory power	very high	medium	low	high	very high	very high
Reproducibility	very good	very good	very good	good	low	good
Labor intensive	yes	yes	no	no	no	no
Fingerprint surveys the entire genome rather than a sub-section	yes	no	no	no	yes	yes
Procedure has been automated	no	yes	no	no	no	no
Cost per sample	high	high	low	low	low	low
Requires use of restriction enzymes	yes	yes	yes	no	no	no
Single methodology applicable to majority of bacterial isolates	yes	yes	no	no	no	yes
Requires sequence knowledge of the organism	no	no	no	yes	no	no

<sup>1</sup> Indicates if technique can easily be completed in less than a day. Both PFGE and ribotyping take several days. However, the automated ribotyping procedure can be completed in one day.

distinguish to the genus- or species-level. During the probe design process, probes can be tested against the extensive database of rRNA sequences using computer models. With the correct design procedure, the resulting probes should have a low cross reactivity. Using this strategy, genus specific probes have been designed and evaluated for detection of *Bacteroides* (Dore *et al.*, 1998), *Bifidobacterium* (Kaulmann *et al.*, 1997), *Lactobacillus* (Sghir *et al.*, 2000) and *Clostridium* (Sghir *et al.*, 2000) from human faecal samples. Species specific probes for bifidobacteria (Yamamoto *et al.*, 1992), *Bacteroides* (Kreider, 1995) and *Lactobacillus* (reviewed, Schleifer *et al.*, 1995) have also been developed.

#### *Pulse Field Gel Electrophoresis (PFGE)*

PFGE essentially means using an electrical pulse system to migrate very large fragments of DNA through an agarose gel (reviewed, Romling *et al.*, 1992). This technology can

be applied to fingerprinting an isolate by digesting its genome into relatively few (5 - 50) large segments and separating them by PFGE. The fragments are obtained by digesting the genome with rare cutting restriction enzymes, which generally have an 8 bp recognition site or a 6 bp recognition site which may be statistically rare for the particular genome. Because the DNA fragments are large, they cannot be manipulated in aqueous solutions or they would be sheared mechanically. Therefore, all manipulations, including DNA isolation and restriction, are carried out on cells embedded in agarose plugs. The restricted fragments in the agarose plug are inserted then into a well in an agarose gel and separated by PFGE based on fragment size. The resulting pattern of DNA fragments is referred to as a restriction fragment length polymorphism (RFLP) (Figure 2a, b) and is highly characteristic of the particular organism. It should be noted that this fingerprint represents the complete genome and has the added

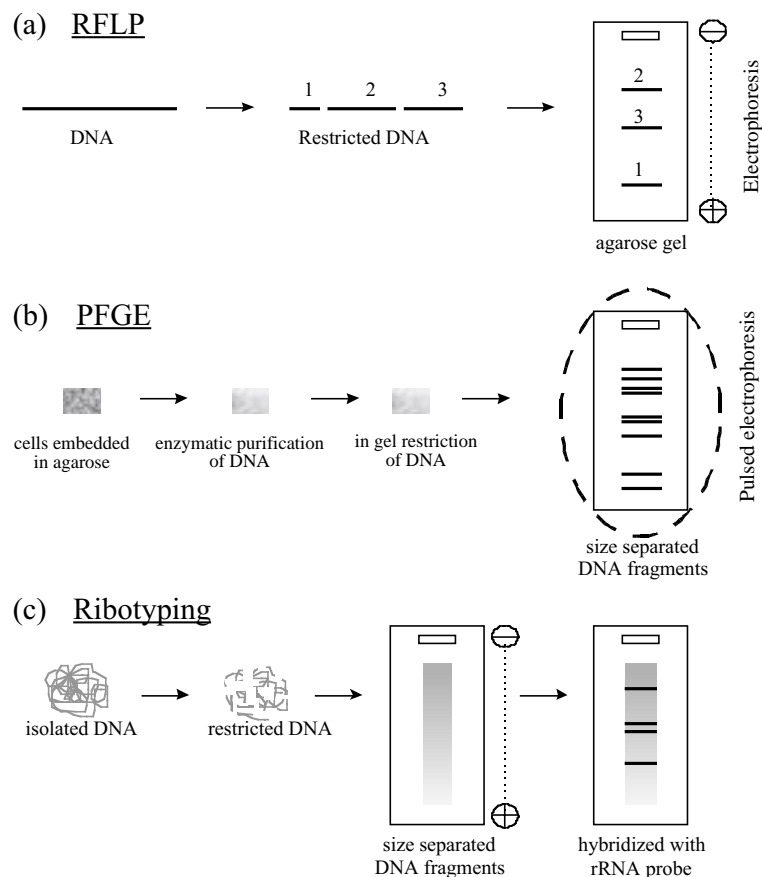


Figure 2. Illustration of commonly used fingerprinting techniques. (a) Demonstration of restriction fragment length polymorphism (RFLP). The DNA fragment is restricted into 3 pieces (1, 2 and 3) by a restriction enzyme. The restricted DNA is then inserted into a well in an agarose gel and the fragments are separated by electrophoresis. The separation is based on fragment size, with the smallest fragments migrating further into the gel. This pattern, termed RFLP, represents a characteristic fingerprint of this DNA fragment. (b) Pulse field gel electrophoresis (PFGE) to separate large restriction fragments (50 - 1,000 kb) into a characteristic RFLP. Whole cells are embedded in agarose and DNA is enzymatically purified and restricted by rare cutting enzymes *in situ*. The agarose plug is then inserted into a well in an agarose gel and the restricted fragments are separated by an electric current which pulses from different angles. (c) Demonstration of ribotyping. DNA is isolated from a cultured isolate, restricted and size separated in an agarose gel. The gel is then hybridized with labeled rRNA probe, which binds to fragments containing copies of the rRNA operon. Following probe detection, fragments with bound probe are visualised, forming a characteristic RFLP.

advantage of detecting specific changes (DNA deletion, insertion or rearrangements) within a particular strain over time. This feature also makes this fingerprinting technique one of the most discriminatory (if not the most) techniques available. It is also very reproducible. Disadvantages of this technique are that colonies need to be cultured to obtain enough cells and that it is technically challenging, as well as labor intensive. The usefulness of the technique has been demonstrated by McCartney *et al.*, (1996) and Kimura *et al.*, (1997) to monitor the prevalence of lactobacilli and bifidobacteria in human fecal samples over a period of time.

#### *Ribotyping*

A ribotype is essentially an RFLP consisting of the restriction fragments from a particular genome which contain rRNA genes. To obtain a ribotype for an organism, it must first be cultured to obtain enough cells for the procedure. Total DNA is then isolated and is totally restricted into multiple fragments, of sizes ranging from < 1 kb to > 20 kb, using a restriction enzyme with a frequently occurring recognition sequence, generally a 6 bp recognising enzyme. The restricted fragments are then separated by agarose gel electrophoresis and subsequently hybridised with a probe targeted to either the 16S, 23S or 5S rRNA genes (Figure 2c). In practice, probes to the 16S rRNA are the most commonly used. The hybridisation can be carried out directly in the gel using in gel hybridisation techniques, or alternatively on a nylon or nitrocellulose membrane following Southern transfer of the DNA from the gel to the filter. Following probe detection, restriction bands containing copies of the rRNA genes are visualised and the pattern of the band sizes represents a characteristic fingerprint. The basis of the technique is that bacteria generally contain multiple copies (up to eight or more) of the rRNA genes throughout their genome, thus enabling the RFLP to be obtained. However, some bacteria contain as few as one copy of rRNA genes, thus limiting the effectiveness of ribotyping for fingerprinting these bacteria. Bacteria with a single copy of the rRNA operon are usually slow growing bacteria. Examples are *Coxiella burnetti*, *Bradyrhizobium japonicum* and *Mycobacterium paratuberculosis*. An advantage of ribotyping is that a single rRNA probe can be used to type all bacteria. It is also very reproducible and its effectiveness for the analysis of human intestinal microflora has been demonstrated (Kimura *et al.*, 1997; Mangin *et al.*, 1995; McCartney *et al.*, 1996). Limitations of this fingerprinting technique are, that it is not as discriminative as PFGE, requires culturing of bacteria and is labor intensive. However, the development of an automated ribotyping instrument (Dupont, Wilmington, DE) has increased the usefulness of this technique for the analysis of large numbers of isolates.

A related fingerprinting methodology is the use of a probe targeted to specific regions within a genome, such as a virulence gene or other unique characteristic of a particular organism. As this targets only a single genetic locus, its discriminatory power is low, but it is highly effective for analysing a population of organisms for specific traits. Probes can also be targeted at other sequences which may exist in multiple copies in a genome, such as insertion sequences (IS elements), thus enabling a characteristic RFLP to be generated. This technique has been used to

fingerprint bacteria (Soldati and Piffaretti *et al.*, 1991), but has not yet been used in the study of the human intestinal ecosystem. However, given the propensity of IS elements in many lactic acid bacteria (Polzin *et al.*, 1993), this may be an applicable technique for this purpose.

#### *RFLP of the 16S rRNA Gene*

This is a rapid technique, which involves amplifying the 16S rRNA gene using the PCR with primers targeted at universally conserved regions within this gene. The resulting amplicon is then restricted with an appropriate restriction enzyme and the resulting restriction fragments are size separated by agarose gel electrophoresis, forming a characteristic RFLP (Figure 2a). The choice of restriction enzyme depends on the particular genus and must be experimentally determined. Kullen *et al.*, (1997b) used this fingerprinting technique to differentiate an ingested bifidobacteria isolate from the indigenous bifidobacteria in human subjects. In this study, the restriction enzyme *HaeIII*, which recognizes the sequence GGCC, was found to generate a characteristic RFLP for this genus. As this is a PCR based technique, it can be carried out on very few cells, thus eliminating the need to culture colonies. This is a major advantage of all PCR based fingerprinting techniques. The discriminatory power of this technique is generally low because of the conserved nature of this gene. However, it has probably the highest reproducibility of all the PCR based fingerprinting techniques.

#### *Multiplex-PCR*

The PCR, which was developed by 1993 Nobel prize recipient Kary Mullis (reviewed, Mullis, 1990), is one of the most useful molecular tools of modern time. In its simplest form, PCR is used to amplify a specific DNA sequence over a billion-fold from a single copy, using a thermostable DNA polymerase (usually *Taq* DNA polymerase), deoxynucleotides (dNTP) and two primers, whose sequence is complementary to either end of the targeted sequence. This is achieved using multiple cycles of the PCR, generally 30 - 40. During each cycle of the PCR, the reaction tube is first heated to ~ 94 °C, which denatures the double stranded template DNA. The temperature is then dropped to < 55 °C (typically), which allows the primers to anneal to their target sequences, and then to 72 °C to enable the *Taq* polymerase to extend from both primers, thus creating a duplicate copy of the DNA region between the two primers. This duplicated region is generally < 5 kb, although it is now possible to amplify much larger fragments. As each duplicated copy becomes template for the next cycle of PCR, the amplification is exponential, where by a single copy is potentially amplified to 2<sup>n</sup> (n = number of cycles). Therefore, in a typical PCR of 35 cycles, ~ 3.4 x 10<sup>10</sup> copies can potentially be generated.

In a multiplex PCR, more than one set of primers is included to enable the simultaneous amplification of a number of target DNA regions (Figure 3a). The more target regions amplified, the more reliable the technique. A disadvantage of the technique is that prior sequence knowledge is required and it is technically challenging to design optimal reaction conditions. It was recently adapted for the reliable identification of human *Lactobacillus* species (Song *et al.*, 2000).

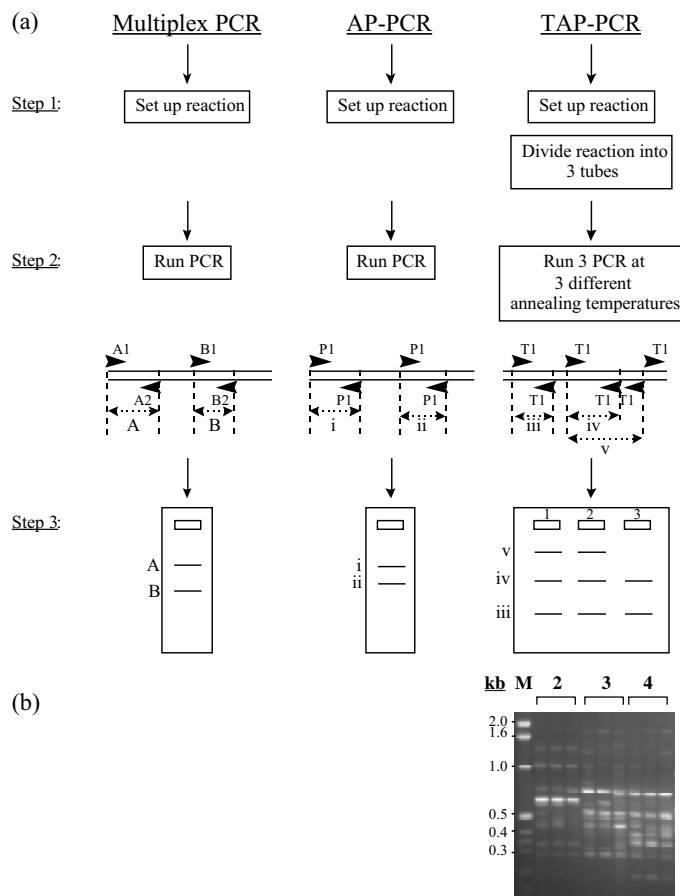


Figure 3. (a) Illustration of three PCR fingerprinting techniques. Step 1: The reaction in each case contains template, primers, dNTP and *Taq* polymerase in an appropriate buffer. The template can be a portion of a colony, which has been disrupted by microwave, physical shearing with glass beads, or other cell disruptive process. The primary difference between the three reactions is the primer(s). The multiplex PCR procedure contains primers A1 and A2, which are specifically targeted at sequences bordering DNA fragment A and primers B1 and B2, which target fragment B. The AP-PCR contains a 10 base primer (P1), whose sequence is arbitrarily chosen. TAP-PCR contains an 18 base primer (T1), whose sequence is based on a conserved region of the 16S rRNA. Step2: The annealing temperatures for both the multiplex and AP-PCR need to be experimentally optimized. The annealing temperatures for the TAP-PCR are 38, 40 and 42°C respectively. DNA regions indicated by dotted lines can be amplified. Step 3: Agarose gel separation of amplified fragments to form characteristic banding patterns. A and B indicate the fragments targeted by the multiplex PCR. Roman numerals indicate the arbitrary primed products. Lanes 1 - 3 indicate the 3 reactions of TAP-PCR. Bands which appear in at least 2 out of 3 lanes are considered reproducible. (b) Example of TAP-PCR fingerprint of *Bifidobacterium breve* ATCC 15701 (lane 2), *B. infantis* ATCC 15697 (lane 3) and *B. infantis* ATCC 15702 (lane 4). The annealing temperatures for each TAP-PCR of each strain were 38, 40 and 42°C respectively. M, 1 kb ladder (BRL).

### Arbitrary Primed (AP) PCR

AP-PCR differs from conventional PCR in that only a single short primer (usually 10 - 12 bases), whose sequence is arbitrarily chosen, is used. To enable the primer to anneal to the template DNA, the stringency of the reaction is reduced, allowing the primer to bind to regions where it exhibits nearest homology. When these primer binding sites are within a few thousand bases and are on opposite strands, the DNA region in between can be amplified (Figure 3a). The more products which are amplified, the more discriminatory the technique. This fingerprinting technique was first described in 1990 and was termed AP-PCR (Welsh and McClelland, 1990) or RAPD (Williams *et al.*, 1990). As this rapid technique is very discriminative and can be applied to organisms for which no sequence information is known, numerous protocols have been developed for many bacterial genera. The major

disadvantage of the procedure is that subtle changes in reaction conditions can change a banding pattern, thus compromising the reproducibility of the technique.

### Triplet Arbitrary Primed (TAP) PCR

The low reproducibility of arbitrary priming results from unintended changes in reaction conditions. By purposely introducing specific changes to the reactions in three otherwise identical reactions, the amplicons which are susceptible to changes in the reactions can be identified. This is the basis of TAP-PCR (Cusick and O'Sullivan, 2000). The triplet reaction is conducted in parallel at three different annealing temperatures (38, 40 and 42°C) and following gel electrophoresis of each reaction, the banding patterns are compared. Bands which are present in at least two lanes are considered resilient to small changes in reaction conditions and are therefore considered in the fingerprint

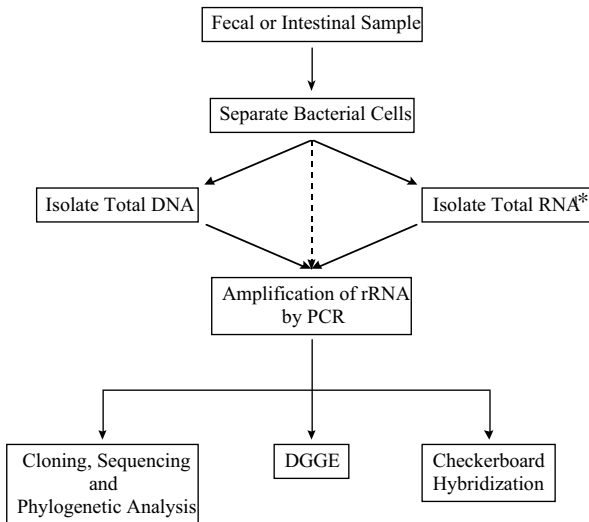


Figure 4. Outline of molecular approaches for culture-independent analysis of the human intestinal microflora.

\* to use total RNA as a template for PCR, a reverse copy of the rRNA gene needs to be generated using the enzyme reverse transcriptase. This process is termed RT-PCR.

analysis (Figure 3a). The technique can be discriminative to the species and strain level (Figure 3b). It maintains the significant advantages of AP-PCR, but increases the confidence limits of the fingerprinting result (Table 1).

#### Culture-Independent Molecular Approaches to Analysing Intestinal Microflora

The advent of culture-independent molecular techniques has in many ways revolutionised the field of microbial ecology. However, to date these tools have not been used to a large extent for the analysis of the human intestinal microflora. A probable reason for the lack of impact these tools have had in the study of this ecosystem, is that unlike many other natural environments, culture techniques have been more successful at identifying a large portion of the intestinal microflora. Recently, these molecular techniques have begun to be directed to the human intestine and should unveil many more mysteries of its complex microbial microflora. The techniques developed to date rely on directly amplifying 16S rRNA from faecal samples using PCR. Generally, faecal samples are first enriched for bacterial cells by differential centrifugation and can be used directly in PCR, or total DNA or RNA can first be extracted. The procedure can target the rRNA genes by using DNA, or cells, in a standard PCR. As rRNA is often thousands of times more plentiful than rDNA genes, total RNA can also be used as template if the enzyme reverse transcriptase is also included. This enzyme can generate a complementary copy of the single stranded rRNA during the first cycle, thus creating a double-stranded template for the PCR. Targeting rRNA, rather than DNA, can be used to preferentially identify bacteria which are metabolically more dynamic, as faster growing bacteria have greater amounts of rRNA.

There are limitations with these rRNA based culture-independent techniques, regarding their estimations of biodiversity in natural habitats. One limitation concerns the disparity in the number of rRNA operons in different bacteria. Clearly, an organism with one copy of rRNA genes

will be under represented compared to organisms with eight or more copies. The disparity is magnified if rRNA is used as the template for the PCR. Another limitation concerns the use of universal primers for the amplification of the rRNA product. These primers are not identically homologous to all bacteria and will not amplify all rRNA products with the same efficiency. This can result in a disparity in the biodiversity in favour of those organisms more conducive to PCR with the primers used. To help control for this limitation, different sets of primers targeting different universally conserved regions within the rRNA can be used.

Following isolation of the amplified 16S rRNA product, there are three strategies that are presently being used for the analysis of the human intestinal microflora (Figure 4): cloning and sequencing of individual rRNA genes; separation of individual rRNA products by denaturing gradient gel electrophoresis (DGGE); and checkerboard hybridization with specific probes.

#### Sequencing of Individual rRNA Genes

The amplification of a 16S rRNA product from a faecal sample results in a heterogenous mix of products, within the amplicon. Cloning the amplicon into a standard sequencing vector can result in a bank of individual rRNA gene clones. These clones can then be sequenced and phylogenetically analysed. This strategy has been fundamental to the discovery of numerous new organisms, as well as to our present taxonomic division of all organisms into *Eucarya*, *Archaea* and *Eubacteria*. Recently it has been applied to the analysis of human intestinal microflora and preliminary indications are that many of the sequences identified were from novel organisms (de Vos *et al.*, 1997; Suau *et al.*, 1999; Wilson and Blichington, 1996). This is extremely noteworthy, as it suggests that culture techniques may not be as effective at analysing the biodiversity of this environment as was generally perceived. The further use of this molecular tool to study the diversity of organisms in the human intestine, will have a major impact on the field of probiotics, as knowledge of the balance of the microflora

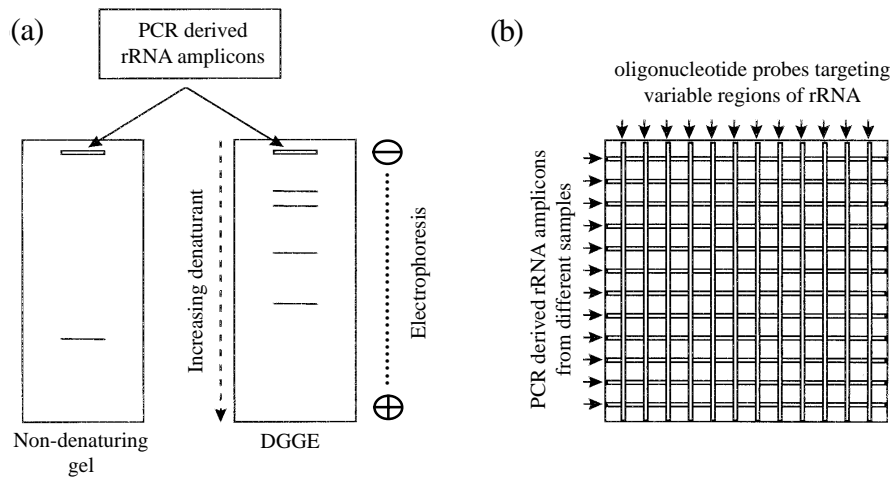


Figure 5. (a) Illustration of density gradient gel electrophoresis (DGGE) of a PCR derived rRNA amplicon from four different bacteria. Gel electrophoresis in a non-denaturing gel results in a single band, whereas electrophoresis through a gel containing increasing concentrations of a denaturant results in the separation of the four different products, based on their sequence dependent melting patterns. Note, to maintain the double stranded integrity of the products, a G/C clamp is generally included in one of the primers used in the amplification. (b) Illustration of the checkerboard hybridization format for analyzing large numbers of samples with multiple probes. Each vertical channel contains a different bound probe and each horizontal channel contains a PCR derived rRNA amplicon from a different individual. In this example, 144 different hybridizations can be carried on one membrane.

in different individuals is paramount to understanding their functional role in intestinal health.

#### Denaturing Gradient Gel Electrophoresis (DGGE)

This procedure can separate individual rRNA genes from the universally amplified product. Although all the individual rRNA species within the amplicon are of the same length, electrophoresis through a linearly increasing gradient of denaturants can separate the products of different sequence (Fischer and Lerman, 1979). The principle is based on the melting of rRNA genes at specific denaturing points based on their sequence. Therefore, each individual sequence will begin to melt at a characteristic denaturing point. The melting changes the conformation of the DNA molecule, slowing its migration through the gel (Figure 5a). Urea and formamide are generally used to form the denaturing gradient. However, temperature can also be used, thus creating a temperature gradient gel electrophoresis (TGGE). When an individual rRNA gene begins to melt, its migration slows and it becomes separated from the PCR amplicon. Further migration of the gene through the denaturing gradient, however, could result in the double-stranded DNA becoming denatured into ssDNA products. To prevent this occurring a GC clamp, consisting of 30 - 50 "G" and "C" bases attached to the 5' end of one of the primers used to amplify the rRNA product, can be used. As G/C rich DNA regions are resilient to melting, this tag can maintain the integrity of the double stranded rRNA genes.

This approach can potentially provide a fingerprint of the complexity of the intestinal microflora of an individual. The use of this technique will enable rapid detection in the makeup of an individual's flora over time (Zoetendal *et al.*, 1998). It can also reveal the presence of new isolates, such as ingested probiotic strains that may be present (de Vos *et al.*, 1997).

#### Checkerboard Hybridization

The ability to rapidly detect certain microbes among an individual's intestinal microflora is very useful, particularly when investigating the distribution of certain species among a large population of individuals. One approach which has been used to detect a probiotic *Bifidobacterium* strain in faeces, was to use species-specific primers directed to the 16S rRNA gene and amplify directly from faecal samples (Kok *et al.*, 1996). An alternative and potentially more sensitive approach would be to use universal rRNA primers to amplify the rRNA amplicon from faeces and subsequently probe the amplicon with species-specific oligonucleotide probes. This approach can be adapted to the analysis of multiple samples with multiple probes at once using checkerboard hybridisation (Socransky *et al.*, 1994). This procedure, which is illustrated in Figure 5b, can potentially enable large numbers of individuals to be screened for specific microflora in a short time. The effectiveness of the technique, however, depends on the specificity of the probes used. The technique also depends on the probes having similar melting temperatures, as all must undergo the same hybridization stringency. At present there are very few probes available with the desired level of specificity and melting stringency. However, future development of more strain-specific probes should render this approach very useful for the analysis of specific microflora in multiple individuals.

#### *In situ* Analysis of Intestinal Microflora

The ability to be able to obtain information on single cells *in situ* in faecal or intestinal samples is very intriguing. This event is now feasible, primarily due to the development of sensitive fluorescent labels, which enable probes to be visualised by fluorescent microscopy. Visualisation of specific strains at the single cell level *in situ*, can be

achieved by prokaryotic *in situ* PCR (PI-PCR) or fluorescent *in situ* hybridization (FISH).

### PI-PCR

*In situ* PCR relies on the amplification of specific gene sequences inside intact cells with primers that have fluorescent tags (reviewed, Long and Komminoth, 1997). This technique has mostly been applied to eukaryotic cells, but has recently been adapted for bacteria. Hodson *et al.*, (1995) developed the PI-PCR to enable *in situ* visualization of individual bacterial cells in natural environments. In this study, primers were developed based on specific genes within the bacteria of interest, and these fluorescent tagged primers were used in a PCR on a glass slide containing a preparation of the cells. Following amplification, individual cells containing the targeted gene could be visualized by fluorescent microscopy. Recently, Tani *et al.*, (1998) used an improved fluorescent label and demonstrated the efficacy of the procedure for visualising specific bacteria at the single cell level in a natural environment. This technique has yet to be applied to the human intestinal microflora. However, it offers tremendous promise, particularly as it can potentially be adapted to determine what individual cells are expressing specific genes.

### FISH

An alternative to *in situ* PCR amplification is to hybridise fluorescent labelled oligonucleotide probes directly to cells fixed on a glass slide (reviewed, Amann, 1995). The fixing process permeates the cells to allow the short probes to access the nucleic acid inside the cell. This hybridization can be carried out on glass slides and the cells with the hybridised fluorescent probe can subsequently be visualized by fluorescent microscopy. Using non-specific probes to the 16S rRNA, FISH has indicated the number of bacteria in human faecal samples is approximately ten-fold higher than estimated using standard culture techniques (Harmsen *et al.*, 2000; Langendijk *et al.*, 1995). The technique has also been evaluated for the detection of specific mRNA species within cells (reviewed, de Vos *et al.*, 1997). This technology, in conjunction with PI-PCR, can potentially reveal what specific genes are expressed by the microflora *in situ* in the human intestine.

### Conclusion

This decade has seen the emergence of numerous molecular approaches for the analysis of different aspects of the human intestinal microflora. Their use, in conjunction with traditional culture methods, has already significantly enhanced our knowledge of this ecosystem. The recent adaptation of culture-independent molecular tools to the human intestinal microflora, offers further potential for revealing a more detailed picture of the true complexity of this unique environment. These techniques also have the potential to explore the functionality of certain microbial traits in the intestine, particularly as *in situ* mRNA detection systems become more sophisticated. The next few years should see the impact of these approaches on our understanding of this ecological niche. The contribution of these studies to the field of probiotics is enormous, as stringent scientific studies are the key to providing the

necessary scientific substantiation for the efficacy of potential probiotic bacteria on intestinal health.

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