

Assessment of Molecular Techniques in Probiotic Study

Beena G Patel, PhD and Rahul Dheer, BS

Address Correspondence to

Beena G Patel, Ph.D.

624 Selma Street

Norristown PA 19401

Phone: 610 272 7168

Email: Beenagpatel@hotmail.com

Key words: Probiotics, Molecular techniques, Human intestinal micro flora

ABSTRACT

The concept of probiotic has been around for more than century. Their impact on human nutrition is still an emerging science, and knowledge of human intestinal micro flora is still growing. To determine the positive effect of probiotic strain, necessary tools are required to monitor the specific strain in the intestines. The addition of molecular methods to conventional microbiological methods for identification of the

ecological makeup of a microbial community has shown significant improvements in the accuracy of identification and enumeration of microbes. This minireview will focus on commonly used molecular techniques for analyzing human intestinal micro flora and some of the techniques used in studying probiotic strains for clinical application.

1. INTRODUCTION

The intestine is a large organ, which consists of a diverse population of more than 400-500 different bacterial species as high as 10¹⁰ to 10¹¹cfu/g (of feces). Predominant species of bacteria are obligate anaerobe and are represented by gram-positive and gram-negative genera [1].

Recently, there has been a renewal of interest in the use of probiotics (living microbial cells that, when consumed, believed to influence the composition of the intestinal micro flora and to benefit the well-being of the host). There is a delicate balance between the host and the colonic micro flora, which not only acts as a protective barrier against infection, but also provides up to 7-10% of the host's daily energy requirements through the fermentation of carbohydrates that escape digestion in the upper gastrointestinal tract [2]. Probiotics have been used therapeutically to modulate immunity, lower cholesterol, treat rheumatoid arthritis, prevent cancer, improve lactose intolerance, and prevent or reduce the effects of atopic dermatitis, Crohn's

disease, diarrhea, and constipation as well as candidiasis and urinary tract infections (UTI) [3]. The normal microbiota of gastrointestinal (GI) tract help synthesize B vitamins, short chain fatty acid, stimulate host immune system and work as a barrier against pathogens [4] However, role and action of individual microbial species or groups present in the GI tract are poorly understood.

Detection of bacteria in feces reflects the bacteria present in the distal portion of large intestine. Therefore, studies of intestinal microbiota usually involve analyses of bacterial community in fecal samples [4]. A bacteriological culture method has been used to analyze fecal micro flora. Even though it has been shown that approximately 88% of the total microscopic counts of bacterial cells can be cultivated from feces when appropriate techniques are employed, it is clear that the remaining micro flora detected by microscopy is uncultivable [3]. The limitation for this cultivation include unknown growth requirement of bacteria, the selectivity of the media, stress

imposed by cultivation procedures, and difficulties with simulating the interactions of bacteria with other microbes and host cells. These limitations require culture independent methods.

The application of molecular methodologies to intestinal micro flora analysis

should facilitate the development of detailed knowledge of microbial ecology of the human colon. This knowledge is essential to derive scientifically validated probiotic strains for clinical applications (Table 1).

Table 1 Molecular techniques used in probiotic study

Probiotic	Method used	Identification
<i>L. rhamnosus</i> DR20 [4]	FISH, PCR-DGGE	Species level
<i>L. rhamnosus</i> GG and <i>B. lactis</i> Bb12 [5]	PCR PCR-DGGE	Species level
<i>Streptococcus thermophilus</i> and <i>Lactobacillus bulgaricus</i> [6]	PCR	Species specific
<i>Lactococcus lactis</i> TC165.5 [7]	PCR(nis A gene) and Conventional method	Strain specific

FISH: fluorescence in situ hybridization

PCR: Polymerase Chain Reaction

DGGE: Denaturing gradient gel electrophoresis

2. PROBIOTICS

Probiotic can be defined as live microorganisms which, when administered in adequate amounts, confer a health benefit to the host [8]. The concept of probiotic has evolved from the work of Metchnikoff (1908). He demonstrated longevity of life due to ingestion of milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus*. Metchnikoff hypothesized that the growth of lactobacillus in gastrointestinal tract would replace other putrefactive bacteria, which would help in

reducing the concentration of toxins in the gut and thus improve health. A number of potential benefits have been proposed; regression of tumor [9], reduction in carcinogen or co- carcinogen [10], reduction in allergy [11], reduction in blood pressure [12], reduction in serum cholesterol concentration [13], decreased duration of diarrhea and increase resistance to infectious diseases [14, 15]. Lactic acid bacteria (LAB) found in fermented food has a long history of safe use. LAB such as *Lactobacillus* and

Enterococcus, are consumed daily. *Lactobacillus acidophilus* has been used safely for more than 70 years. Some strains of Streptococcus and Enterococcus show the properties of LAB, *Streptococcus thermophilus* being the only strain currently used in fermented dairy products.

It cannot be assumed that all probiotic strains are safe. Before incorporating a new strain into any products, it should be carefully tested for safety and efficacy. Three approaches can be pursued to assess the safety of probiotics; a) the origin of microbial strain. b) the safety and stability aspect related to - activity, viability of product, invasive potential, resistance to low pH, gastric juice, bile acid, pancreatic juice, colonization and *in vivo* survival, and c) the functional and physiological aspects, including adherence to intestinal walls, antagonism to pathogen, stimulation or suppression to immune response, selective stimulation of beneficial bacteria and suppression of harmful bacteria and/or clinical side effects. It is very necessary to

3. TRANSITION FROM CONVENTIONAL CULTIVATION TO MOLECULAR TECHNIQUES

Microbial ecologists generally compare the microscopic count to total viable count to obtain an estimate of cultivability. It has been assumed that total viable counts are typically lower than total microscopic count, due to the number of dead cells [21]. Nucleic acid based studies indicated that the majority of bacteria in a

know not only the positive effect of a probiotic but also its side effects.

At present, there is no general guideline available for testing safety of probiotic. *In vitro* methods, animal models, and human subjects can be used to determine safety of probiotics. Some recommendations are mentioned in Donohue *et al.*, [16]. The safety of probiotics has been questioned in recent reviews and clinical reports as in the cases of human bacteremia associated with the presence of LAB [17, 18]. In the clinical treatment of gastrointestinal disorders of both children and adults, a variety of probiotic bacteria have been used. These include conditions where mucosal layers are harmed by antibiotic or radiotherapy, acute diarrhea of bacterial or viral origin, and prevention of gut colonization by pathogen [19]. No evidence of opportunistic infection was seen in these studies. Recent study by Saxelin *et al.*, showed that the clinical isolates of *Lactobacillus* from bacteremic patients were not involved in human infections [20].

variety of ecosystem are different compared to those bacteria described in cultures. Therefore, it is rational to assume that the differences between microscopic and total viable counts are due to an inability to cultivate the cultures. This has led to an extensive development and application of molecular techniques to the study of complex microbial ecosystem. (Table 2)

Table 2 Summary of current techniques used to study complex microbial ecosystems [22]

Methods	Uses	Limitations
Cultivation	Isolation; "the ideal"	Not representative; slow & Laborious
16S rDNA sequencing	Phylogenetic Identification	Laborious; subject to PCR biases
DGGE/TGGE/TTGE	Monitoring of community/population shifts; rapid comparative analysis	Subject to PCR biases; Semiquantitative; identification requires clone library
T-RFLP	Monitoring of community shifts; rapid comparative analysis; very sensitive; potential for high throughput	Subject to PCR biases; semiquantitative; identification requires clone library
SSCP	Monitoring of community/population shifts; rapid comparative analysis	Subject to PCR biases; semiquantitative; identification requires clone library
FISH	Detection; enumeration; comparative analysis possible with automation	Requires sequence information; laborious at species level
Dot-blot hybridization	Detection; estimates relative abundance	Requires sequence information; laborious at species level
Quantitative PCR	Detection; estimates relative abundance	Laborious
Diversity microarrays	Detection; estimates relative abundance	In early stages of development; expensive
Non-16S rRNA profiling	Monitoring of community shifts; rapid comparative analysis	Identification requires additional 16S rRNA-based approaches

PCR: Polymerase Chain Reaction

DGGE: Denaturing Gradient Gel Electrophoresis

TGGE: Temperature Gradient Gel Electrophoresis

TTGE: Temporal Temperature Gradient Gel Electrophoresis

T-RFLP: Terminal restriction fragment length polymorphism

SSCP: Single Strand Conformational Polymorphism

FISH: Fluorescence in Situ Hybridization

3.1 Genetic labeling of lactic acid bacteria

In order to monitor a particular strain, insertion of an extra DNA label has been used, but at the same time, introduces concern for human consumption. For example, a foreign DNA containing antibiotic resistance that is incorporated into the particular strain (as was done for lactic acid bacteria) may cause the spread of this resistance gene to the normal bacterial flora. "Food-grade" markers have been an alternative approach for markers of targeted strains. One example is the labeling of a lactic acid bacterium with a plasmid-encoded green fluorescent protein gene, which is placed under an inducible promoter in order to observe the

phenotypic change [23]. Although the approach of food-grade marker possesses much less of a potential threat to the consuming organism, genetically engineered organisms, in general, is not favored by the legislation. An alternative method of genetic labeling of bacterial strains is the alteration of one or few base pairs, which causes a change in the genotype, but not the phenotype of a strain also known as silent mutation. The strain can therefore be identified on a genetic basis rather than by a phenotypic characteristic.

3.2 Commonly used Molecular typing method

3.2.1 Ribotyping method

To identify and classify bacteria based upon differences in the rRNA, ribotyping is used. It generates a highly reproducible and precise fingerprint that can be used to classify bacteria by the genus at the species level. DNA is extracted from a colony of bacteria digested with an appropriate restriction endonuclease and resulting discrete-sized fragments are separated in an agarose electrophoretic gel, transferred to hybridization membrane, and probed with a radiolabeled ribosomal RNA sequence. Several fragments in the restriction digest hybridize the probe since bacteria have multiple copies of rRNA operons in their chromosome. This method has been routinely used in water analysis laboratories.

3.2.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is a technique commonly used to amplify a specific region of DNA or RNA of a target species for a range of applications. For example, this molecular technique is used for the development of DNA probes, identification of genetic disorders [24], forensics [25], feces analysis [26], and analysis of genetic lineages. The procedure involves the use of primers, which adhere to a recognized sequence of the denatured single stranded DNA, which will ultimately produce a complementary segment of the DNA with the assistance of DNA polymerase and deoxynucleotide triphosphates. PCR involves three main steps; denaturation of DNA (into two single strands), annealing of the primers to

specific DNA sequences, and synthesis of the complementary strand using the initial DNA strand as the blueprint. This process is repeated 35 or more times in an automated thermocycler, which results in multiple copies of the targeted DNA segment of the particular strain. The result is segments of DNA of varying lengths (base pairs) which can be separated and analyzed by Gel Electrophoresis, which separates the DNA segments based on size, as the base pairs travel from negative to positive field through a semi-permeable medium (agarose). This technique is useful when comparing two different strains, which will produce a different pattern of DNA segments (based on the deviations of base sequences between the two experimental strains). This technique, known as DNA fingerprinting, has a variety of applications from bacterial strain identification to criminal investigation (forensics). The technique used for bacterial fingerprinting in such applications as feces analysis relies on the use of 16s rRNA instead of genomic DNA, as the template for PCR.

3.2.3 Real time PCR

As previously mentioned, the theoretical basis of PCR is to amplify a certain segment of DNA or RNA in order to help distinguish genetic variations between two strains (genotyping). However, there are two main types of PCR used today, Traditional PCR and Real-Time PCR. As was discussed earlier, with respect to the traditional PCR method upon completion of the set cycles, the product is analyzed by electrophoresis, which separates the bands based on the physical properties of the gel as well as the DNA/ RNA segments (size,

charge, density of gel, etc). The limitation of Traditional PCR is

that the samples are analyzed after the completion of cycles. Therefore, the end product being analyzed is the end product of the reaction and therefore is known as end-point detection. Real- Time PCR differs from this traditional method by analyzing the progress of the reaction at each cycle in log phase without disrupting the progress of the reaction. Real-Time PCR is commonly used in viral and gene quantifications [27], drug therapy efficacy, DNA damage measurements, pathogen detection, and genotyping [28].

3.2.4 Polymerase chain reaction-enzymelinked immunosorbent assay (PCR-ELISA)

PCR-ELISA is a molecular technique of screening for specific genetic sequences obtained from PCR products. This procedure involves the labeling of the PCR product with digoxigenin (a plant steroid hormone), which bind to a specific antibody (anti-digoxigenin antibody) which contains horseradish peroxidase, enzyme commonly linked with an antibody for detection of a specific antigen (digoxigenin in this case). The PCR product is tagged with biotin that has a strong affinity to a protein known as streptavidin. Therefore, the streptavidin is contained within the micro-titer plate, which the PCR product binds via biotin. The antibody-enzyme complex then bind to the PCR product which is specific for the antigen digoxigenin, and therefore as the substrate 3, 3', 5, 5' – tetramethylbenzidine reacts with the horseradish peroxidase, and a resulting blue color can be measured with a spectrophotometer [29]. One of the most useful

aspects of the PCR-ELISA is differentiating between polymerase chain reaction products generated from a common set of primers that contain sequence variations, i.e., sequence variation between the primers. As the assay plates are relatively inexpensive to prepare, capture probes can be added, deleted, and modified readily.

3.2.5 Repetitive element sequence-based PCR (rep-PCR)

Repetitive element sequence-based PCR (rep-PCR) is a new typing method that differentiates microbes by using primers complementary to interspersed repetitive consensus sequences that enable amplification of diverse-sized DNA fragments consisting of sequences between the repetitive elements [30]. Multiple amplicons of different sizes are fractionated by electrophoresis and the resulting DNA fingerprint patterns, specific for individual bacterial clones, are compared. Numerous studies have shown that the application of rep-PCR using oligonucleotide primers based on the repetitive extragenic palindromic (REP) elements (REP-PCR) or on the enterobacterial repetitive intergenic consensus (ERIC) sequences (ERIC-PCR) has been successful in typing a variety of bacteria [31].

3.2.6 Pulse field gel Electrophoresis

PFGE is a method for characterization of isolate based on restriction fragment length polymorphisms of chromosomal DNA. Bacteria are embedded in agarose and lysed in situ, using the restriction endonuclease enzyme, which cuts chromosomal DNA infrequently. Slices of agarose are added to agarose gel and the restriction fragments are separated by a gel

electrophoresis. Each reorientation of the electric field relative to the gel, provides a separation of the smaller DNA fragment from the larger DNA fragment.

Currently, there are three models that attempt to describe the behavior of DNA during PFGE [32], the biased repetition model (BRM), the chain model, and, most recently, the bag model [32, 33].

3.2.7 Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis is a new method for characterization of isolates, which is based on relative electrophoretic mobilities of larger number of water-soluble enzymes. Rate of migration of protein during electrophoresis are determined by amino acid sequences. Variation in sequences reflects mobilities of the proteins. The study of genetic relationships of 42 strains of *Klebsiella pneumoniae* and 24 strains of *Klebsiella oxytoca* demonstrated the reliability of the method [34].

3.2.8 Random amplification of polymorphic DNA

The RAPD technique employs 10 base pairs of random primers, which hybridize at random sites to initiate DNA polymerization in the polymerase chain reaction (PCR). The DNA is cut into many segments of a specific length, which can be measured using gel electrophoresis. The number and location of these priming sites vary between strains. The electrophoretic pattern of the DNA fragments amplified by PCR gives a fingerprint characteristic of each bacterial strain.

4. CONCLUSION

In conclusion, current molecular approaches along with conventional methods have significantly enhanced our knowledge of the complexity of human intestinal micro flora. However, there are some questions still unanswered, such as type, number, complexity and interaction of intestinal microbes. We need to improve our understanding of synergistic action between commensal bacteria and a human host, of the mechanisms by which beneficial bacterial species maintain or improve health, and

References

1. Tannock G. W. 1995. Normal micro flora. An introduction to Microbes inhabiting the human body. *Chapman and Hall London*, UK.
2. Sofia, K, Tuohy, K., Tuohy and Glenn R. G. 2000. The human gut flora in nutrition and approaches for its dietary modulation. *Nutrition Bulletin*. 25, 223-231.
3. O'Sullivan, D. J. Methods of analysis of the intestinal micro flora, 1999. In G. W. Tannock (ed.), *Probiotics: a critical review*. Horizon Scientific Press, Wymondham, United Kingdom.. p. 23-44.
4. Tannock, G. W., Munro, . K. , Harmsen, H. J. M. G. Wellng, W. J. Smart, and P. K. Gopal. 2000. Analysis of the Fecal Micro flora of Human Subjects Consuming a Probiotic Product Containing *Lactobacillus rhamnosus* DR20. *Appl Environ Microbiol*. 66. 2578–2588.
5. Satokari, R.M., Vaughan, E.E. . Smidt, H., Saarela, M J. Matto, and W.M. de Vos 2003. Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. *Syst. Appl. Microbiol*. 26, 572-584.
6. Sonja, L., Karsten,D., and Knut J.H.2001. Survival of *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* in the Terminal Ileum of Fistulated Göttingen Minipigs. *Applied and Environmental Microbiology*. 67, 4137-4143.
7. Klijn, N., Weerkamp, A.H. and de Vos, W.M.1995. Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. *Appl. Environ. Microbiol*. 61, 2771-2774.

of the change mechanism for the whole micro flora not just change in few species. The contribution of these molecular techniques to the field of probiotic study is enormous and can provide significant information about the efficacy of potential probiotic bacteria on intestinal health

Acknowledgements

Authors are grateful to Dr. Heena Patel and Dr. Surender Dheer for helpful suggestions during manuscript preparation.

8. FAO/WHO report, 2001.http://www.mesanders.com/probio_report.pdf
9. Kato, I., Kobayashi, S., Yokokura, T. and Mutai, M. 1981. Antitumor activity of *Lactobacillus casei* in mice. *Gann*. 72, 517-523.
10. Goldin, B. R., Swenson, L., Dwyer, J., Sexton, M. and Gorbach, S. L.1980. Effect of diet and *Lactobacillus acidophilus* supplements on human fecal bacterial enzymes. *J Nat. cancer Inst*. 64, 255-261.
11. Isolaurie, E., Majamaa, H., Arvola, T., Rantala, I., Virtanen, E. and Arvilomni, H. 1993. *Lactobacillus casei* GG reverses increased intestinal permeability induced by cow milk in suckling rats. *Gastroenterol*. 105, 1643-1650.
12. Sawada, H., Furushiro, M., Hirai, K., Motoike, M., Watanabe, T. and Yokokura, T. 1990. Purification and characterization of antihypertensive compound from *Lactobacillus casei*. *Agric. Biol. Chem*. 54, 3211-3219.
13. Gililand, S. E. and Walker, D. K.1990. Factors to consider when selecting culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *J Dairy Sci*. 73, 905-911.
14. Saaverdra, J. H., Bauman, N. A., Oung, I. , Perman, J. A., and Yolken, R. H. 1994. Feeding of *Bifidobacterium bifidus* and *Streptococcus thermophilus* to the infants in the hospital for prevention of diarrhea and shedding of rotavirus. *Lancet*, 344, 1046-1049.
15. Fernandes, C. F., Sahani, K. M., and Amer, M.A 1987. Therapeutic role of dietary

- lactobacilli and lactobacilli fermented dairy products. *FEMS Microbiol. Rev.* 46, 343-356.
16. Donohue, D. C., Deighton, M., Ahokas, J.T., Salminen, S.1993. Toxicity of lactic acid bacteria. In: Salminen S, von Wright A, eds. Lactic acid bacteria. Marcel Dekker Inc, New York . 307-313.
17. Aguirre, M.and Collins, K., 1993 Lactic acid bacteria and human clinical infection. *J Appl Bacteriol.* 75, 95-107.
18. Adams, M.R., Marteau, P. 1995. On the safety of lactic acid bacteria from food. *Int J Food Microbiol.* 27, 263-264.
19. Lee, Y-K., Salminen, S.1995. The coming of age of probiotics. *Trends in Food Sci Technol.* 6, 241-245.
20. Saxelin, M., Rautelin, H., Chassy, B., Gorbach, S.L., Salminen, S., Mäkelä, P.1996. Lactobacilli and septic infections in Southern Finland during 1989-1992. *Clin Infect Dis.* 22,564-566.
21. Apatalahti, J.H.A., Kettunen, A., Nurminen, P. H., Jättilä, H. & Wolben, W. E. 2003. Selective plating underestimates abundance and shows differential recovery of bifidobacterial species from human feces. *Appl. Environ. Microbiol.* 69, 5731-5735.
22. Erwin, G., Zoetendal, C., Collier, T., Satoshi, K., Roderick, I. M., and Rex Gaskins, H.2004 Molecular Ecological Analysis of the Gastrointestinal Microbiota. *A Review. J. Nutr.* 134, 465-472.
23. Geoffroy, M.C., Guyard, C., Quatannens, B., Pavan, S., Lange, M., Mercenier, A. 2000. Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. *Applied and Environmental Microbiology* 66, 383-391.
24. Wang, R.F., Cao, W.W., Cerniglia, C. E. (1997). PCR detection of Ruminococcus spp. in human and animal faecal samples. *Molecular and Cellular Probes.* 11, 259-265.
25. Almoguera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N., Perucho, M. (1988) *Cell.* 53, 549-54. 26. Kok, R. K., De Waal, A., Schut, F., Welling, G. W., Weenk, G., Hellingwerf, K. J. (1996). Specific detection and analysis of a probiotic Bifidobacterium strain in infant feces. *Applied and Environmental Microbiology* .62, 3668-3672.
27. Giorgio, G., Francesca, B., Claudia, F., Stefania, D., Elisabetta, M., Marialuisa, Z., and Monica, M. 2004. Calibrated Real-Time PCR for Evaluation of Parvovirus B19 Viral Load. *Clinical Chemistry.* 50, 759-762.
28. Betty, H., Robertson, and Janet K.A. Nicholson., 2005. New Microbiology Tools for Public Health and Their Implications *Annual Review of Public Health.* 26, 281-302.
29. Hatano, H., Kawashima, H., Ogoose, A., Hotta, T., Endo, N. 2001. A PCRELISA assay for the detection of disseminated osteosarcoma cells in a mouse metastatic model. *J Orthop Sci.* 6, 269-275.
30. Olive, D. M., and Bean, P. 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* 37, 1661-1669.
31. Alves, A., Santos, O., Henriques, I. and Correia, A. 2002. Evaluation of methods for molecular typing and identification of members of the genus Brevibacterium and other related species. *FEMS Microbiol. Lett.* 213, 205-211.
32. Chu, G. 1990. Pulsed-field electrophoresis: theory and practice. In *Methods: A Companion to Methods of Enzymology. Pulsed-Field Electrophoresis* (B. Birren and E. Lai, eds.) *Academic Press*, San Diego. 1, 129-142.
33. Chu, G. 1991. "Bag model for DNA migration during pulsed-field electrophoresis." *PNAS* 88, 11071-11075.
34. Marie-Laure, C., Jean-Louis, Pons., Richard, S., and Jean-Pierre, M.1994. Electrophoretic Transfer from Polyacrylamide Gel to Nitrocellulose Sheets, a New Method to Characterize Multilocus Enzyme Genotypes of Klebsiella Strains. *Appl Environ Microbiol.* 60, 26-30