SIV-1

# PROBIOTICS AND HEALTH

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## Abstract

The ability of microbial strains to confer health benefits to human and animal hosts is not a feature of many organisms. Lactic acid bacteria are the most commonly used bacteria applied as probiotics and there is now strong evidence that certain strains confer tangible benefits to the host. In terms of preventing infection, the ability of probiotic lactobacilli to colonize the tissue site, even temporarily, and inhibit growth and adhesion of pathogens, has been documented. Using molecular tools, such as RAPD and DGGE, probiotic organisms can be tracked through the intestine and in the vagina, and their impact on the flora assessed. Arguably, strains *L. rhamnosus* GG and GR-1 are the most studied probiotic strains in terms of human application to the gut (GG and GR-1) and vagina (GR-1). Combined with *L. fermentum* RC-14, GR-1 provides a two-pronged therapeutic for the intestine and urogenital tract. Care in manufacturing and distribution is essential to ensure that optimal doses of probiotics are accessible to consumers and patients.

## INTRODUCTION

The term probiotics (pro meaning bio or life) refers to living microorganism(s) administered to promote the health of the host by treating or preventing infections owing to strains of pathogens, or to conferring upon the host other health benefits (1). The organisms are Generally Regarded As Safe (GRAS) by government regulatory bodies, and an analysis of studies carried out with probiotics has shown conclusively their safety in humans (2). Primarily, probiotic strains come from *Lactobacillus, Bifidobacteria, Saccharomyces* and *Enterococcus species.* The term biotherapeutic Agents_ has also been used in the context of microorganisms which have a therapeutic effect on humans (3), but probiotics is the preferred term.

**What is so special about probiotic organisms?**

Humans are exposed to hundreds of different microbes throughout their life, and indeed live because of those which inhabit the intestine, skin, nose, throat and urogenital tract. However, only a

select group of organisms would be considered for active ingestion or application to the host. Thus, most of the organisms used as probiotics have not been found to be pathogenic to the host under normal circumstances. Nevertheless, under the right conditions, such as an immunosuppressed host and direct inoculation into bloodstream, even lactobacilli can cause infection (4).

Organisms such as alpha streptococci, enterococci and *E. coli* have been used as probiotic agents with some degree of success in the nasopharynx (5), gut (6) and bladder (7) respectively. These organisms have been selected because of their ability to compete with pathogens for receptors at sites such as the tonsils not readily amenable to lactic acid bacteria, or to block specific receptors sites such as in the bladder where *E. coli* colonize. However, there is some concern that these species can potentially infect the host or not function as probiotics through mishandling in passage (5), acquisition and transfer of multi-drug resistant genes (8) and acquisition of virulence factors or production of undesired side effects (9).

While each probiotic strain and intended site of application will present differently, in general probiotic organisms should fit into their environment and function in a way that is beneficial to the host. This might include use of their fermentation pathways for digestion of foods, given that these organisms have developed or adapted metabolic pathways to ferment milk, cheese and other material. In general such reactions are not adversarial to the host. On the other hand, organisms such as *Pseudomonas* and staphylococci have adapted to soil, plants, skin and surfaces where they utilize proteases and toxins for survival. Thus, in the human and animal host they can be toxic.

This paper will now explore some of the investigative tools used to study the properties of probiotic strains and methods to track their colonization.

## MATERIALS AND METHODS

### In vitro selection methods to identify and assess probiotic strains

*Adherence.* Adherence is considered important to maintain probiotic strain at target sites. Bacterial adherence to vaginal epithelial cells can be evaluated by adding $10^5$ cells/ml vaginal epithelial cells (collected by swabbing) to $10^8$ cfu/ml bacteria (lactobacilli grown in MRS broth or agar under strict anaerobic or microaerophilic conditions; pathogens grown in brain heart infusion agar) and incubating for 1 hour at 37°C. Net adhesion is enumerated on 50 Gram stained cells by deducting control counts on cells incubated with PBS alone (10). Similar studies can be carried out using cells in monolayer culture lines.

*Cell surface hydrophobicity* The hydrophobic properties of strains can influence adhesion, although in our experience both hydrophilic and hydrophobic strains can attach to cells (11). The

property can be measured using a water contact angle method.

*Inhibition of adhesion of pathogens.* Several mechanisms have been proposed for competitive exclusion or bacterial interference of pathogens (12). This includes steric hindrance and receptor site blockage. The former is assessed by pre-incubating cells with lactobacilli for 1 hour or more, then challenging with the pathogen strains. The pathogens can be $^{3}H$ labeled or fluorescently tagged to identify. Coincubation of both strains can also be used (13) and arguably this is a better test because *in vivo* surfaces are rarely free of bacteria, and competition is a constant occurrence. Certain strains of lactobacilli and oral streptococci produce biosurfactants that inhibit attachment of microbes to surfaces. By strict definition, these should decrease the liquid surface tension as measured by axisymmetric drop shape analysis by profile (ADSA-P)(14). However, the biological activity of microbial inhibition does not seem to be based primarily on surface tension changes, and so the ability to inhibit adhesion of pathogens such as *Enterococcus faecalis* 1131 to polystyrene can be used to assess the activity (15). Within the biosurfactant mixtures of proteins and carbohydrates, certain molecules could be particularly effective in the interference process. For example, a 29 kDa protein produced by *L. fermentum* RC-14 binds to collagen and inhibits enterococcal adhesion (16, 17) in wound infection caused by *S. aureus* (18). While there is no set level of inhibition of adhesion that has been found to correlate to *in vivo* effectiveness, strains that do not inhibit 75% *in vitro* adhesion of pathogens likely would not be selected.

*Pathogen growth inhibition* Production of organic acids, bacteriocins, hydrogen peroxide and other factors has been shown to be inhibitory to growth of some pathogens. Likewise, pathogens probably produce substances derogatory to probiotic strains, but to date studies of this type have not been done. The organisms can be tested for $H_2O_2$ production using the tetramethylbenzidine agar plate method (19), and for inhibition of growth of *E. coli* Hu734 by the agar overlay method (10).

*Molecular Techniques* For specific identification of organisms within a body site, nucleic acid based techniques have been developed and are rapid, reliable and more sensitive than classical strain identification methods (20). These include:

*Pulsed field gel electrophoresis* (PFGE) which involves the use of rare cutting restriction enzymes to digest the microbial genome into relatively few (5-50) large DNA segments. In this way, a DNA fingerprint, which is highly characteristic of the particular organism, is obtained and referred to as a restriction fragment length polymorphism (RFLP).

*Ribotyping* involves the restriction of the total genome with an endonuclease, separation of the DNA fragments by agarose gel electrophoresis and subsequent hybridization with a probe to either 16S, 23S or 5S rRNA genes. Bacteria generally contain multiple copies of rRNA genes throughout their genome and thus a number of fragments of different sizes will hybridize to the probe, giving a

characteristic fingerprint. The restriction fragments are transferred to nitrocellulose membranes and hybridized with 16S and 23S rRNA $^{32}$P-labelled probes to yield distinct ribotype patterns.

*DNA probes* are labelled synthetic oligonucleotides of defined sequence that can specifically hybridize to a target complementary sequence. For species-specific probes, oligonucleotides are commonly directed towards regions of 16S and 23S rRNA. Once a suitable probe has been designed and labeled, DNA or RNA is extracted from the microorganism or sample of interest. The probe is hybridized to the extracted nucleic acids which are immobilized on nylon or nitrocellulose filters or hybridization can be performed directly on living cells. The label, which can be radioactive, enzymatic or fluorescent is then used to detect specific targets. By using fluorescent labelled rRNA probes, it is possible to detect microorganisms *in situ* (FISH - fluorescent in situ hybridization).

*Polymerase chain reaction (PCR)* can also be used to subtype bacteria within a genus or species by employing 16S rDNA, arbitrary oligonucleotides, species-specific DNA and intergenic spacers, among others, as primers. Direct extraction of PCR-quality bacterial DNA from tissue samples is possible.

*Randomly amplified polymorphic DNA (RAPD)* PCR employs short arbitrarily chosen primers which bind to genomic DNA at randomly occurring complementary target sequences under low stringency annealing conditions. Resultant DNA fingerprints differentiate strains in a multispeciated specimen, but pure cultures of the microorganisms are required.

*Density gradient gel electrophoresis* The system uses a hypervariable sequence region of the 16S rRNA gene amplified by PCR. The mixture of 16S DNA fragments is subjected to DGGE to separate the fragments and obtain a profile of the microbial community. A profile is generated by differences in the distance into the gradient where denaturation occurs and migration ceases, of the 16S fragments that have different nucleotide base compositions (21). Species-specific primer pairs allow analysis of certain organisms within a mixture without the need for culture. This technique has been used successfully to profile the gut flora before and after probiotic intake (22).

*Additional microscopy and other analytical tools.* Bacteria adherent to tissues can be viewed under electron microscopy following fixation in 5% glutaraldehyde and cacodylate buffer (0.1M, pH 7.2) with 0.15% ruthenium red for 2 hours at room temperature, embedding and sectioning. Labeled probes (such as antibody conjugated to fluorescein or immunogold secondary antibody) can be used to identify the species in the samples. More sophisticated microscopy tools include confocal laser scanning microscopy (CLSM) and deconvolution microscopy for acquisition of three-dimensional (3-D) images of biofilms. The DeltaVision software (IRIX based softWoR$_x$_ version 2.5) is capable of reducing out-of-focus fluorescence.

*Proteomics* In order to examine proteins produced by probiotic organisms, and of potential

therapeutic benefit, SELDI WCX-1 ProteinChips (Ciphergen Biosystems Inc., Palo Alto, CA, USA) can be used along with time of flight mass spectroscopy. For example, cCollagen types III (bovine skin, Sigma) and VI (human placenta, Sigma) has been used on chips to identify collagen binding proteins (17).

*Other properties* Not all probiotic strains are selected for their ability to prevent or cure infection. Some might be designed to lower serum cholesterol, break down carcinogens in the gut, modulate immune function, inhibiting pathogen translocation across enterocyte layers, or other purposes (23-26).

## RESULTS AND CLINICAL OUTCOMES

The resultant probiotic, selected from the above criteria, should be able to adhere to host surfaces, inhibit growth and attachment of pathogens, and not carry multi-drug resistance plasmids. In essence, a strain must have been shown to possess mechanisms of action whereby they could confer benefits to the host. That does not mean that non-adherent strains will not be functional in other ways *in vivo*, but it makes correlation with clinical outcomes more difficult to explain.

If the intent is for strains to be developed for specific clinical use, such as disease treatment, there are generally four phases of human study required (27). Even if a probiotic is only being developed as a dietary supplement, consideration should be given to clinical testing, as it adds credibility to the product.

Phase 1 is essentially to test safety of the product, and this usually necessitates small patient numbers. Lactobacilli probiotics are generally regarded as safe, as long as they are pure and do not contain pathogens as too often happens with some products (28).

Phase 2 is to show that a drug has a specific desired effect. A study design should determine the dose effect, with the aim of finding the least toxic and best outcome for the patient.

Phase 3 involves proving that the drug is efficacious compared to the gold standard treatment that would normally be given for this disease. The optimal dosage should be tested against a placebo, in a randomized, double-blinded fashion.

Phase 4 is often regarded as a post-marketing phase, whereby the drug is approved by the regulatory agency, but its longer term effects are monitored. This can be done by surveys, interviews with patients and doctors, sales analyses and other methods.

In the past, probiotics have been criticized for lack of efficacious data, but in recent times, this has been corrected and studies have indeed shown clear health effects with certain strains including reducing diarrhea, day care infections, allergy, inflammation and urogenital infections (29-34). Our

experience with *L. rhamnosus* GR-1 and *L. fermentum* RC-14 has shown that they colonize the vagina well, survive passage through the intestine, possess factors antagonistic to urogenital and intestinal pathogens, and prevent recurrence of infection. This represents the first two-pronged therapeutic probiotic for men and women health.

## PRODUCT QUALITY

For oral probiotics, survival during gastrointestinal transit (through the acidic stomach and bile) is important to convey health benefits (35). Many studies have demonstrated intestinal transit of potentially probiotic microorganisms, with recovery from faecal samples usually used as an indication of survival (36-39). However, bacterial numbers can reach levels as high as $10^{10}$-$10^{12}$ CFU/g in the large intestine and therefore passage is only the first stage in making a therapeutic difference in the tract. A minimum dose of $10^8$ cfu appears necessary (40).

It is critical that each strain is prepared in a condition which optimizes viability and shelf-life, and that product labels specify content and accurate past-due dates (41). Dairy delivery vehicles, especially yogurt and milk, are currently the most common means to sell human probiotics (42).

In summary, many excellent studies have now proven that certain microbial strains can confer health benefits to the host. Specific application of proven strains to intended site of action should provide new strategies for disease treatment, and health restoration and maintenance.

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