PROBIOTIC: AN OVERVIEW FOR SELECTION AND EVALUATION

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ABSTRACT
Recent studies have increased our understanding on the mechanistic basis of the proposed probiotic health effects. Although they are effective in curing the disease for which they are prescribed, the effect on the indigenous gut flora may persist even after cessation of the treatment. The concern about undesired side effects of use of antibiotics as therapeutic agents produced a climate in which both consumer and manufacturer are looking for alternatives. Probiotics are being considered as effective alternative. Well designed human studies have demonstrated that specific probiotic strains have health benefits in the human population. These have led to a wide acception of the probiotic concept. However, current probiotics have not been selected for specific purposes. Novel methods to select and characterize target-specific probiotic strains are thus needed. In addition to the traditional selection procedures, in recent years, knowledge on intestinal microbiota, nutrition, immunity and mechanisms of action has increased dramatically and can now be combined with genomic data to allow the isolation and characterisation of new target- or site-specific probiotics. We should expect to see new, third generation probiotics emerging in the near future and also new selection criteria further defining the targets of future probiotics.

Keywords: Lactobacillus, Intestinal microbiota, Screening, Probiotics

INTRODUCTION
Fermented products containing living microorganisms have been traditionally used to restore gut health. Such utilization of live microorganisms to improve host health forms the basis of the probiotic concept. Probiotics have been defined as live microorganisms which, when administered in adequate amounts, confer a health benefit to the host. This definition suggests that safety and efficacy of probiotics have to be demonstrated for each strain and each product. Selected strains (fig 1), mainly belonging to the genera Lactobacillus and Bifidobacterium, are increasingly being used as probiotics. After ingestion they must overcome biological barriers, including acid in the stomach and bile in the intestine, to reach their place of action in order to exert their health-promoting effects. To produce therapeutic benefits, a sufficient number of viable microorganisms must be present throughout the entire shelf life of the product. However, these organisms often show poor viability in market preparations. The basis for assessing probiotic efficacy in humans requires the understanding of probiotic strains, each of which is unique and different. Thus, the strain properties and characteristics should be well defined, and studies using closely related strains cannot be extrapolated to support each other. The assessment has to be based on a valid scientific hypothesis. Working hypotheses can be supported by in vitro or in vivo studies with animal models. However, the most important studies for efficacy assessment are carefully planned and monitored clinical studies in humans, conducted preferably by at least two independent research groups in different locations. Recent developments have provided a mechanistic basis of the proposed health effects, and sound human intervention studies have demonstrated that a significant disease risk reduction can be achieved through the use of probiotics in specific human populations. The probiotic concept is now generally accepted. However, current probiotics have not been selected for specific purposes but they rather are 'all-purpose' probiotics. Novel methods to select and characterise target-specific probiotic strains are therefore needed.

Need
The protective flora, which establishes itself in the gut, is very stable but the delicate balance of this beneficial microflora can be disrupted by a number of natural and manmade factors. Pharmaceuticals and natural antibiotic can save lives, but they are indiscriminate killers; they destroy both harmful and beneficial bacteria. Antacids change the pH of the digestive tract; creating an environment favorable for the growth of pathogenic bacteria and yeast. Excessive alcohol, large amounts of sugar, NSAID’s, radiation, chlorine and fluorine, bacterial dysentery, stress, high meat, high fat diets also disturbs normal bacterial flora. When harmful or bad bacteria invade us they take-up residence on the lining of our intestinal tract. They multiply and spread taking over more and more intestinal area. This leads to a loss of the good bacteria. The minor symptoms that occur are blotting, bowel wind, indigestion, constipation, diarrhea, etc. These symptoms continue, and start to occur most days and we get used to them being there. Clearly there is now a shortage of beneficial bacteria- i.e. a probiotic deficiency. It is important to replace the beneficial bacteria and know how to encourage their growth, while minimizing the expansion of the unfriendly ones.

Advantages of Probiotics
• Help to maintain normal cell growth and regeneration.
• Maintain regularity and normal healthy stool consistency.
• Maintain healthy intestinal pH.
Mechanisms of Probiotic action
Several mechanisms of action have been proposed to explain the beneficial effects of probiotics. Nevertheless, our knowledge on probiotics’ mechanisms of action is only preliminary, and it must be taken into consideration that these mechanisms may be multifactorial and each probiotic strain may have specific functions affecting the host. In general, probiotics do not colonise the human intestinal tract permanently, but some strains are able to transiently colonise and to modulate the indigenous microbiota. Specific probiotic bacteria have been reported to modulate local and systemic immune responses. Although the mechanisms of immune modulation are not fully understood it is known that bacterial components are recognized by the immune system through their interaction with specific Toll-like receptors resulting in the modulation of immune responses. The specific receptors implied in some of these interactions have been reported. Probiotic bacteria may also counteract inflammatory processes by stabilizing a healthy microbiota and thus improving the intestine’s permeability barrier. In addition to influencing gut microbiota and immune system, other mechanisms of probiotic action have been proposed, such as inhibition of pathogens by competition for nutrients and attachment sites or by production of antimicrobial substances, reduction of cholesterol levels through deconjugation of bile salts or binding of toxins and carcinogens preventing their absorption. However, mechanisms of probiotic action are multifaceted, and each probiotic may have specific functions affecting the host. It is obvious that an understanding of the cross-talk between the intestinal microbiota and its host expands our conceptions of the relationship between microbiota and health. With regard to this it is clear that genomic information is of great importance. Genomic data on some members of the human intestinal microbiota have provided information on how specifically these bacteria are adapted to the gut. Some microorganisms have been shown to modulate glycosylation of the intestinal mucus and to induce the production of antimicrobials by the mucosa, revealing proposed mechanisms whereby intestinal microbes may influence the gut micro-ecology and shape the immune system.

Genomic research has also provided information about the adhesive mechanisms present in probiotic microorganisms which comprise a basis both for populating the gut and for communicating developmental signals to specific areas and sites of the gut mucosa. In addition, factors related to the immunomodulatory ability of specific strains have been found and bacteriocin operons have been identified. The knowledge on genome sequences gives an idea of the potential properties of those microorganisms but fails to give information about the actual in vivo situation. In this sense, genomic research is important as it provides tools such as DNA microarrays, to unravel the in vivo functions of probiotics and allowing monitoring of the effect of probiotic consumption on host’s genes expression.

Clinical indications
Many types of diarrheal illness with many different causes, disrupt intestinal function. The ability of Probiotics to decrease the incidence or duration of certain diarrheal illnesses is themost substantiated health effect of probiotics. *Lactobacillus* is safe and effective as a treatment for children with acute infectious diarrhea. Probiotics have also been shown to decrease traveler’s diarrhea and recurring colitis due to *Clostridium difficile*. Consumption of high
levels (~10^9 per day) of certain strain of probiotic may shorten the duration or decrease the incidence of certain diarrheal illnesses.

**Lactose Intolerance**

Many people throughout the world suffer from lactose intolerance due to a congenital deficiency of the enzyme β-galactosidase resulting in the inability to digest lactose. People of northern European descent are unique in retaining the ability to produce the lactase digesting enzyme, lactase, into adulthood. Consumption of lactose by those lacking adequate levels of lactase can result in symptoms of diarrhea, bloating, abdominal pain and flatulence. These symptoms are due to the undigested lactose reaching the large intestine and being fermented by the colonic microbes. These microbes can produce gases and products that lead to watery stool. *L. acidophilus* and *Bifidobacteria* have been shown to improve digestion of lactose.

**Constipation**

Some of the first clinical trials carried out with lactobacilli were related to their effect on constipation. Recently there have been recorded encouraging results with the use of Lactophillus milk for the treatment of constipation.

**Hypertension**

Most of the people are estimated to have hypertension or elevated blood pressure. Evidence suggests that consumption of certain lactobacilli or milks fermented with lactobacillus lead to decrease in elevated blood pressure. Two triptides generated by growth of *lactobacillus helveticus* during the production of milk yielded an antihypertensive effect. These triptides were shown to inhibit angiotensin converting enzyme.

**Cancer**

Cancer is caused by mutation or activation of abnormal genes that control the cell division. Most of these cells do not result in cancer since normal cell usually out-compete abnormal ones. Also, the immune system recognizes and destroys most abnormal cells. Many processes or exposures can increase the occurrence of abnormal cell. Among the potentially risky exposures are chemical exposures. Cancer causing chemicals (carcinogens) can be ingested or generated by metabolic activity of microbes that live in the gastrointestinal system. It has been hypothesized that probiotic culture might decrease the exposure to chemical carcinogens by (1) detoxifying ingested carcinogens, (2) altering the environment of intestine and thereby decreasing populations or metabolic activities of bacteria that may generate carcinogenic compounds, (3) producing metabolic products (e.g. butyrate) which improve cell’s ability to die when it should die [a process known as apoptosis or programmed cell death]. (4) producing compounds that inhibit the growth of tumor cells, or (5) stimulating the immune system to better defend against cancer’s self proliferation.

**AIDS and Leukemia**

The immune system provides the primary defense against the microbial pathogens that have entered our bodies. The immune system is extremely complex, involving both cell based and antibody-based responses to potential infectious agents. Immunodeficiency can result from certain diseases or, to a lesser extent from more normal conditions such as old age, pregnancy, or stress. Autoimmune diseases can also occur due to redirected immune system activity. Probiotic cultures have been shown in variety of test systems to stimulate certain cellular and antibody function of the immune system. The structures of the catalytic core of two HIV-1 encoded enzymes play a crucial role in the retroviral cycle: integrase and RNase H. It has been reported to be that of HIV-1 integrase and RNase H. It has been reported to be present in retroviral particles and to enhance cDNA synthesis. Some topoisomerase inhibitors have been shown to be active on integrase. Moreover, topoisomerase, integrase and RNase H are inhibited by G-rich oligonucleotides. A G-quartet structure is necessary for integrase, but not for topoisomerase inhibition. This suggests that prototype structures can be exploited to develop inhibitors of two related enzymes, such as the RNase H and integrase activities of HIV-1 RT.

**Vaginosis**

The vagina and its microflora form a finely balanced ecosystem. Disruption of this ecosystem can lead to a microbiological imbalance and symptoms of Vaginosis. It leads to serious conditions such as pelvic inflammatory disease; pregnancy related complications (low birth weight babies) and increased susceptibility to AIDS infections. Lactobacilli predominate in the healthy vagina and the lack of lactobacilli (especially those producing hydrogen peroxide) is correlated with Vaginosis. The lactobacilli are thought to maintain a favorable vaginal pH in the acidic range and to inhibit pathogen via the production of hydrogen peroxide.

**Kidney stones**

High levels of oxalate in the urine is risk factor for the development of the kidney stones. Utilization of oxalate by intestinal microbes limits its absorption. Anaerobic preparation containing bacteria was able to degrade oxalate in vitro and was shown to reduce oxalate fecal excretion. Manipulation of gut flora with the right probiotic bacteria may have a positive impact on gastrointestinal tract oxalate levels and may decrease oxalate absorption.

**Elevated blood cholesterol**

Cholesterol is essential for many functions in the human body. It acts as a precursor to certain hormone and vitamins and it is a component of cell membranes and nerve cells. However elevated levels of total blood cholesterol or other blood lipids are considered risk factors for developing coronary heart diseases. Although human synthesize cholesterol to maintain minimum levels for biological functioning, diet also is known to play a role in controlling serum cholesterol levels, although the extent of influence varies significantly from person to person. Probiotic cultures have been evaluated for their effect on serum cholesterol levels.

**Pharmaceutical Approach**

Different strains are being used in the pharmaceutical industries shown in table 1. Many marketed preparations are available containing LAB as well as combination with drugs like Amoxycillin, Cloxacillin, Ampicillin, Clotrimazole, Cefixime etc. The various dosage forms include tablets, capsules, powders, microcapsules, ORS Powders, Liquid preparations etc.

**Intestinal microbiota**

Gut microbiota has been recognized as a significant factor influencing our health and well-being. The human gastrointestinal tract harbours a complex collection of microorganisms which form a specific individual microbiota for each person (fig 2). This specific microbiota is dependent on the environment and genetic factors. The total number of microbes in the intestinal tract can be estimated at the level of 10^12 bacteria per gram intestinal contents. The interaction between microflora and host plays important functions in the human body being necessary, for instance, for the establishment of oral tolerance 32-33 or the maintenance of intestinal homeostasis 34. The healthy microbiota may be considered a good source of future probiotics. Most microbiota studies used culture-dependent methods. Conventional culture-dependent methods have some limitations such as low sensitivity, time consuming, bias introduced due to the culture and recovery of only the cultivable species of the intestinal microbiota. These facts can lead to the overestimation of some species and the underestimation of others. Thus, during the last decade developments in molecular biology have led to the application of fast and reliable alternative culture independent methods 35. Taking into account the considerations on
intestinal microbiota and health it is obvious that understanding the cross-talk that occurs between intestinal microbiota and its host promises to greatly expand our knowledge about the relationship between intestinal microbiota and health. There is an increasing amount of information indicating that specific aberrancies in intestinal microbiota may make us more vulnerable for intestinal inflammatory diseases and other diseases beyond the intestinal environment. It is likely that some aberrancies even predispose us to specific diseases. Unfortunately, we are still far from knowing the qualitative and quantitative composition of the intestinal microbiota and which factors govern its composition in an individual. Assessment of microbiota effects of probiotic consumption may be important for selection and evaluation of new probiotics with specific targets in microbiota.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus NCFM®</td>
<td>Rhodia, Inc. (Madison, WI)</td>
</tr>
<tr>
<td>L. acidophilus SBT-20621</td>
<td>Snow Brand Milk Products Co., Ltd. (Tokyo, Japan)</td>
</tr>
<tr>
<td>B. longum SBT-29281</td>
<td></td>
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<tr>
<td>L. rhamnosus R0011, L. acidophilus R0052</td>
<td>Institut Rosell (Montreal, Canada)</td>
</tr>
<tr>
<td>L. acidophilus LA-1, L. paracasei/CRL 431</td>
<td>Chr. Hansen (Hørsholm, Denmark)</td>
</tr>
<tr>
<td>B. lactis Bb-12</td>
<td></td>
</tr>
<tr>
<td>L. casei Shirota1, B. breve strain Yakult1</td>
<td>Yakult (Tokyo, Japan)</td>
</tr>
<tr>
<td>L. casei Immunitas</td>
<td>Danone (Paris, France)</td>
</tr>
<tr>
<td>L. fermentum RC-14, L. rhamnosus GR-1</td>
<td>Urex Biotech (London, Ontario, Canada)</td>
</tr>
<tr>
<td>L. johnsonii La-1 (same as NCC533 and formerly L. acidophilus La-1)</td>
<td>Nestlé (Lausanne, Switzerland)</td>
</tr>
<tr>
<td>L. plantarum 299V, L. rhamnosus 271</td>
<td>Probi AB (Lund, Sweden)</td>
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<tr>
<td>L. reuteri SD2112</td>
<td>Biogไnia (Raleigh, NC)</td>
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<td>L. rhamnosus GG1</td>
<td>Valio Dairy (Helsinki, Finland)</td>
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<td>L. rhamnosus LB21, Lactococcus lactis LT1</td>
<td>Essum AB (Umeå, Sweden)</td>
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<tr>
<td>L. salivarius UCC118</td>
<td>University College (Cork, Ireland)</td>
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<tr>
<td>B. lactis HN019 (DR10), L. rhamnosus HN001 (DR20)</td>
<td>Fonterra (Wellington, New Zealand)</td>
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<tr>
<td>L. acidophilus LB</td>
<td>Lacteol Laboratory, (Houdain, France)</td>
</tr>
<tr>
<td>L. paracasei F19</td>
<td>Medipharm (Des Moines, Iowa)</td>
</tr>
</tbody>
</table>

**Fig. 2: Distribution of Nonpathogenic Microorganisms in Healthy Humans**

**Technological aspects of probiotics**

**Characteristics of good probiotics**

- It should be strain, which is capable of exerting a beneficial effect on the host animal, e.g. increased growth or resistance to disease.
- It should be non-pathogenic and non-toxic.
- It should be present as stable cells, preferably in large numbers.
- It should be capable of surviving and metabolizing in the gut environment, e.g. resistant to low pH and organic acids.
- It should be stable and capable of remaining viable for long periods under storage and field conditions.

**Strain identification**

A reliable probiotic product requires correct identification of the bacterial species used and the announcement on the label of the species actually present. This is important as a number of recent reports have shown that the identity of recovered microorganisms does not always correspond to the information stated on the product label. Strains should be identified using currently available molecular methods, such as 16S rDNA sequencing or DNA/DNA hybridisation and up-to-date taxonomical nomenclature.

**Physiological characteristics**

Several methods are available to study physiological characteristics of probiotic strains. Carbohydrate fermentation and enzymatic activity profiles have been used widely in this regard. It is important to select the specific substrates or enzymatic activities relevant to the expected functional effects of the strain. Other
specific tests such as the ability to hydrolyse bile salts or to produce antimicrobial substances may be interesting depending on the proposed use of the strain.

**Tolerance to gastrointestinal conditions**

The viability of probiotic strains is considered to be important in order to ensure their expected functionality. After ingestion, these bacteria must overcome two main biological barriers, the acidic environment of the stomach and bile secreted in the duodenum. To guarantee their survival during passage through the gastrointestinal tract, probiotic strains are primarily screened for their tolerance to acid pH and bile. The effect of gastrointestinal conditions on probiotics' survival has been assessed and different bacteria show different levels of tolerance. Different techniques have been used for this purpose. The lack of standard procedures for evaluation of tolerance to gastro-intestinal conditions makes comparison difficult. In general, tolerance to gastrointestinal conditions is low; as a consequence, several methodologies including those based on stress adaptation mechanisms of probiotic bacteria are being investigated as possible strategies for the enhancement of their acid and bile resistance.

**Adhesion**

Adhesion to the intestinal mucosa is often an additional selection criterion for probiotic strains. It may increase the retention time of a probiotic in the gut and puts bacteria and epithelial cells in close contact. Different methods and models have been used for probiotics adhesion assessment including adhesion to intestinal mucus and adhesion to epithelial cells. The effects of gastrointestinal conditions (pH, bile, digestive enzymes) and the effects of acid and bile resistance acquisition on the adhesion of probiotic bacteria have been documented. Highly adhesive strains such as *Bifidobacterium lactis* Bb12 and *L. rhamnosus* GG are effective in preventing and treating acute diarrhoea in infants. In spite of the lack of definitive proof, some studies appear to indicate a relationship between in vitro adhesion and in vivo colonization. Some modulation of the immune system and these have been recently related to certain beneficial effects of probiotics. Moreover, adhesion of probiotic bacteria at the target sites would result in an enhanced exposure to probiotics at the place of action, achieving the desired responses even at a lower dosage.

**Growth and activity**

Multiplication in the intestinal tract would increase the size of probiotic population and the concentration of its metabolic products, thereby increasing their ability to exert a beneficial effect. It is not clear whether probiotics can grow in the intestinal environment. Until now, commercial probiotics have not been able to permanently colonise the gut. However, some probiotics are able to attach to the mucosa and can be recovered in biopsies much longer than in faeces. Thus, mucosal studies are needed to complement faecal recovery studies, as faeces probably do not provide a reliable representation of the microbiota at mucosal level. It is important to evaluate the in situ activity of probiotic strains; in this sense, recent molecular biology developments provide interesting methods for this assessment. The production kinetics of certain metabolites can be relevant; for instance, acid and peroxide production by bacteria are linked to growth, but secondary metabolites that are nongrowth linked may be produced when cells are not multiplying. These metabolites may have a role in the probiotic effect of the strains.

**Viability in probiotic products**

It is widely accepted that to have a positive effect on health, probiotics in foods should remain viable during storage and gastrointestinal transit. It has been reported that viable bacteria alleviated symptoms in eczema patients whereas administration of heat-inactivated cells was associated with adverse gastrointestinal symptoms, underlining the importance of viability. Different minimum levels of probiotics in probiotic products have been reported; however these levels are likely to depend on the specific strain used. Dose–response studies are needed in the assessment of probiotic strains, to determine the effective level of bacteria in one given product. Traditionally, plate counts have been used for the enumeration of viable bacteria. However, modern methods have shown that in some cases probiotic bacteria may lose their culturability but may be still viable. Many studies have shown that bacterial viability is not a simple question, and viable but not cultivable or dormant bacteria may exist in probiotic products. Measuring viability of probiotic bacteria is thus a complex issue, reliable determination may require a multi-method approach and standard methods should be developed in this area. The health effects of so-called dormant probiotic bacteria are yet to be determined, and there is great demand for future research on this topic. These facts should be taken into consideration when assessing probiotic bacterial viability.

**Efficacy of probiotic strains**

Living microorganisms have long been used as supplements to restore gut health at times of dysfunction. However, as different probiotics may interact with the host in different manners, the data available for the most common probiotics and their health benefits need to be assessed, on a strain by strain basis, before any health-related product claims could be approved. It is important to understand that all probiotic strains are unique and different and their properties and characteristics should be well defined. It is important to clearly identify each strain using modern methodology and to make the strains available for all research groups participating in the assessment of health effects and mechanisms. Knowledge of the mechanisms is an important factor, complemented with target functions and biomarkers that are accepted as relevant to the state of health and well-being or reduction of risk of disease. The hypothesis can be supported by *in vitro* or *in vivo* studies using animal models. However, the most important studies are clinical studies in human subjects, conducted preferably by at least two independent research groups in different locations. Multicenter studies offer one good solution for this assessment. Dose–response studies are needed and the best vehicle for the administration of the probiotic strain should be selected. In this regard it is important that the designed products fulfil the adequate nutritional requirements of the target population. Specific protocols for probiotics efficacy assessment are needed. Although specific protocols for probiotics are not available, the application of the efficacy assessment protocols normally used in the pharmaceutical industry has provided a standard for probiotic studies. By using this approach it can be said that certain specific probiotics have scientifically proven benefits which can be attributed to specific products. Other reported probiotic health-related effects are only partially established, requiring more data from larger double-blind, placebo-controlled studies before reaching firm conclusions.

**Safety of probiotics**

Safety assessment is an essential phase in the selection and evaluation of probiotics. Few probiotic strains have been specifically tested for safety but the long history of safe consumption of some probiotics could be considered the best proof of their safety. Although some lactobacilli and bifidobacteria have been associated with rare cases of bacteraemia, usually in patients with severe underlying diseases, the safety of members of these genera is generally recognised due to their history of safe use and lack of toxicity. On the other hand, the low incidence of infections attributable to these microorganisms, together with a recent study showing that there is no increase in the incidence of bacteraemia due to lactobacilli in Finland despite the increased consumption of probiotic lactobacilli, support this hypothesis. With regard to other bacteria such as enterococci, *Saccharomyces boulardii*, *Clostridium butyricum* or some members of the genus *Bacillus* which have been used as probiotics, the situation is more complicated even when they have been used for some time, requiring further case by case assessment. FAO and WHO and the countries represent requested guidelines (outlined in fig 1) and recommendations for the criteria and methodologies required.
to identify and define probiotics and establish the minimum requirements needed to accurately substantiate health claims.

Fig. 4: FAO and WHO Guidelines for Probiotics

**CONCLUSIONS**

Knowledge accrued regarding the intestinal microbiota, nutrition, immunity, mechanisms of action and specific diseases has increased dramatically and can now be combined with genomic data to allow the development of a new generation of probiotic strains for site- and disease specific action. Methodologies have become more specific in intestinal microbiota deviation assessment. This allows us to identify further aberrancies and deviations. At the same time, it will allow us to develop probiotics which counteract the observed deviations or reduce the risk of developing deviations in the future. Thus, harnessing the above described new and novel methodologies to isolation and characterizing new target- or site-specific probiotics has become easier and more focused. We should expect to see new, third generation probiotics emerging in the near future and also the selection criteria further defining the targets of future probiotics.

**REFERENCE**


