Original Article

Safety of probiotics

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New species and more specific strains of probiotic bacteria are constantly being sought for novel probiotic products. Prior to the incorporation of novel strains into food or therapeutic products a careful evaluation of their efficacy is required and an assessment made as to whether they share the safety status of traditional food organisms. Food organisms intrinsic to the production of traditional foods have been arbitrarily classified as safe in the absence of scientific criteria. Evidence for the safety and efficacy of probiotics has until recently been largely anecdotal or based on relatively little, and often poorly designed research. The demonstration of efficacy in probiotics offers vast opportunities for the development of human and veterinary products. The introduction of a new probiotic culture demands that it be at least as safe as its conventional counterparts. Many bacteria are being tested to find a putative probiotic, yielding conflicting data, sometimes for the same organism. Comparisons between studies and organisms cannot be readily made because of non-standardized dosing procedures. Information is not readily available on the equivalence of formulations for different probiotic preparations. There is vigorous debate on what constitutes appropriate safety testing for novel probiotic strains proposed for human consumption. Conventional toxicology and safety evaluation is of limited value in assessing the safety of probiotics. The addition of novel bacterial strains to foods and therapeutic products requires reconsideration of the procedures for safety assessment. This paper provides an overview of these issues.

Key Words: probiotic, safety, bacteraemia, clinical trial, guidelines, efficacy.

Introduction

Probiotics are generally accepted as being live organisms which when administered in adequate amounts confer a health benefit on the host.[1] The demonstration of efficacy in probiotics offers vast opportunities for the development of human and veterinary products: new species and more specific strains of bacteria are constantly being sought for novel probiotic products. Their safety cannot be assumed. The incorporation of novel bacterial strains into foods and therapeutic products requires reconsideration of the procedures for safety assessment. Probiotic products which claim specific nutritional, functional or therapeutic characteristics blur the boundaries between food, dietary supplement or medicine, posing challenges for regulators. Their efficacy should be carefully assessed and an evaluation made as to whether they share the safety status of traditional organisms.

Many of the organisms to which we ascribe probiotic effects have their origins in dairy products and fermented foods. They have been consumed as constituents of these foods without apparent ill effect for centuries. Probiotic organisms are commonly from the genera *Lactobacillus* and *Bifidobacterium*, with some strains of *Enterococcus* and *Saccharomyces* species being amongst the exceptions. They are not specifically adapted to survive in the gastrointestinal tract and are generally regarded as safe because of their long history of use. They have been associated with disease rarely, usually as opportunistic infections in people with predisposing conditions.[2] The use of 'history of safe use' as a criterion for the safety of food organisms is an arbitrary classification. Probiotics consumed in foods and dietary supplements do not have to comply with more rigorous guidelines for probiotics which claim amelioration or prevention of disease in clinical use.

Evidence for the efficacy and safety of probiotic organisms has until recently been largely anecdotal or based on relatively little, and often poorly designed research. Lactic acid bacteria (LAB) and yeasts intrinsic to the production of traditional foods have been accepted as safe without any real scientific criteria, partly because they are normal commensal flora, and because of their presence for generations presumably without adverse effect.

The concept of genetic manipulation of bacteria to achieve a specific probiotic function has appeal. Consumer resistance to genetically modified organisms (GMO) in foods is such that GMO probiotics are unlikely in the near future, with the possible exception of clinical applications. Steidler *et al.*,[3] and Kaur *et al.*,[4] have treated mice models with genetically modified bacteria, to prevent colitis and enhance the efficacy of anti-tumour therapy respectively. Probiotics can thus be designed to

produce potent bioactive chemicals. However extrapolation from proof of principle in a murine model to therapeutic applications for humans will require stringent safety assessment for proposed GMO probiotics.

Existing guidelines for probiotic safety

Conventional toxicology and safety evaluation is of limited value in assessing the safety of probiotic bacteria. Vigorous debate continues on what constitutes appropriate safety testing for novel probiotic strains proposed for human consumption. In recent years several organisations have formulated approaches to assess the safety of probiotics.

The Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food[1] proposed a framework consisting of strain identification and functional characterisation, followed by safety assessment and Phase 1, 2 and 3 human trials. It recommended that probiotic foods be properly labelled with the strain designation, minimum numbers of viable bacteria at the end of shelf-life, storage conditions and manufacturer's contact details. The Working Group further proposed that the use and adoption of the guidelines should be a prerequisite for calling a bacterial strain 'probiotic'.

The Working Group considered the minimum tests required to characterise safety are:

- Determination of antibiotic resistance patterns
- Assessment of metabolic activities (e.g. D-lactate production, bile salt deconjugation)
- Assessment of side-effects during human studies.
- Post-market epidemiological surveillance of adverse incidents in consumers.
- If the strain being evaluated belongs to a species known to be either a mammalian toxin producer or to have haemolytic potential, it must be tested for toxin production or haemolytic activity.

The European Food Safety Authority (EFSA) has proposed a scheme based on the concept of qualified presumption of safety (QPS), defined as 'an assumption based on reasonable evidence' and qualified to allow certain restrictions to apply.[5] The scheme aims to have consistent generic safety assessment of micro-organisms through the food chain without compromising safety standards. Individual evaluations would be limited to aspects particular to the organism, such as acquired antibiotic resistance determinants in lactic acid bacteria. QPS status would not apply to a micro-organism that commonly causes pathogenicity. A micro-organism would not necessarily be considered a potential pathogen where there are infrequent reports of clinical isolates from severely ill people.

Broadly the characteristics to be evaluated for QPS approval are:

- Unambiguous identification at the claimed taxonomic level.
- Relationship of taxonomic identity to existing or historic nomenclature.
- Degree of familiarity with organism, based on weight of evidence.
- Potential for pathogenicity to humans and animals.

- The end use of the micro-organism. This will influence any qualifications imposed, depending on whether the organism is to be directly consumed; is a component of a food product not intended to enter the food chain, but which may adventitiously; or is used as a production strain in a product intended to be free of live organisms.

A discussion of aspects of these guidelines follows.

Taxonomic identification

The introduction of a new probiotic culture demands that it be at least as safe as its conventional counterparts. Is the strain associated with safe food use, an intestinal strain isolated from humans, a strain isolated from animals, or a genetically modified strain? The safety of a putative novel probiotic is contingent on its unequivocal identification at the genus, species and strain level as probiotic effects are strain specific. Sophisticated phenotypic and molecular techniques are available to identify species and discriminate between closely related strains. Correct taxonomic identification of both species and strain is a safety issue for quality control of the product, consumer or prescriber information, diagnosis and appropriate treatment of suspected clinical cases and epidemiological surveillance of the exposed population.

The taxonomy of lactic acid and other bacteria has changed significantly with the advent of genetic methods of classification. Strains previously thought to be dissimilar have merged, while other strains have been added or reassigned to different genera. The persistent use of incorrect or non-existent species names on product labels despite taxonomic reassignation is a significant issue for the safety and credibility of probiotics.

Yeung et al.,[6] used partial 16S rDNA sequencing to identify named commercial strains obtained directly from the manufacturer and found discrepancies in 14 of 29 species designations. Lourens-Hattingh and Viljoen[7] concluded that probiotic cultures in South African yogurt were little more than a marketing tool upon finding the initial counts of *Bifidobacterium bifidum* in three different sources of commercial yogurts were lower than the therapeutic minimum. Weese[8] identified isolates from eight veterinary and five human probiotics to find accurate descriptions of organisms and concentrations for only two of the 13 products.

Temmerman et al.,[9] found that of isolates from 55 European probiotic products, 47% of food supplements and 40% of dairy products were mislabelled. The food supplements yielded either no viable bacteria (37%) or significantly lower counts than the dairy products, contradicting the concept that health benefits derive from the presence of a minimum concentration of live probiotic bacteria.

In six products, all species isolated conformed to the label description; in 19 products they differed from those listed. *Enterococcus faecium* was isolated in such high numbers that contamination was unlikely to be the source. Only two of the 22 food supplements purporting to contain *Lactobacillus acidophilus* did. Bifidobacteria were isolated from five of 27 products claiming to contain them, despite the use of different selective media. The

organism most frequently claimed to be in, and isolated from dairy products was *L. acidophilus*, though it was not necessarily found where claimed.

These and other studies[10,11] demonstrate continued inaccurate identification and mislabelling of probiotic products. Inaccurate nomenclature has no scientific or regulatory validity, misinforms or confuses the consumer, and compromises the safety of the product. Consumers are entitled to expect that the label on a probiotic product accurately reflects its contents: the organism is what it purports to be, it is present alive in a specified concentration range for a stated period, and the suggested serving size contains sufficient organisms to achieve the claimed benefit.

Pathogenicity

It is essential that a probiotic should not cause infection. This is a significant issue where the intestinal barrier is immature as in infants; where its integrity is impaired from radiotherapy, antibiotic treatment or disease; and in immunocompromised states, such as human immunodeficiency virus (HIV) infection. With advances in medical care, an increasing proportion of the community may at some time be immunocompromised, or at risk of opportunistic infection.

Lactobacillus species in general are thought to have low pathogenicity or be opportunistic pathogens in immunocompromised individuals or those with serious underlying disease. It has been suggested that *Lactobacillus rhamnosus* in particular warrants surveillance because it is associated with more cases of bacteraemia than other lactobacilli. *L. rhamnosus* is among the most common *Lactobacillus* species in the human intestine so this may be relative to its extensive presence in the intestine.[12]

Two clinical cases have been reported in which a lactobacillus indistinguishable from an ingested probiotic strain has been identified in association with infection. A 74 year old woman with hypertension and diabetes mellitus developed a liver abscess in association with pneumonia and pleural empyema. She had a history of drinking a probiotic milk containing *L. rhamnosus* GG and a strain indistinguishable from that was isolated from the abscess.[13] A 67 year old man with mild mitral regurgitation developed endocarditis after dental extractions. His blood cultures were positive for a strain of *L. rhamnosus* indistinguishable from that in probiotic capsules he chewed.[14]

Wolf *et al.*,[15] assessed the safety of probiotic *Lactobacillus reuteri* in a double-blind, placebo-controlled study in HIV adults, and found the organism to be well tolerated with no significant safety problems. In a review of probiotic safety Borriello *et al.*,[16] found no published evidence that immunocompromised patients had an increased risk of opportunistic infection from probiotic lactobacilli or bifidobacteria.

Antibiotic resistance and susceptibility

There is potential for viable probiotics to colonise the intestinal tract and transfer genetic material. Whether resistance genes can be transferred by a probiotic organism to the endogenous flora, or vice versa, and the

impact this would have on antibiotic treatment has yet to be elucidated.

Lactic acid bacteria are naturally resistant to many antibiotics by virtue of their structure or physiology. In most cases the resistance is not transferable and the species are also sensitive to antibiotics in clinical use. However it is possible for plasmid-associated antibiotic resistance to spread to other species and genera. The transmissible resistance of enterococci to glycopeptide antibiotics such as vancomycin and teicoplanin is of particular concern, as vancomycin is one of the remaining effective antibiotics for the treatment of multidrug-resistant pathogens.[12]

Antibiotic resistance mechanisms, their genetic nature and transfer characteristics of resistance determinants have been studied comparatively recently in anaerobic bacteria. It has been shown that the plasmid which encodes for macrolide resistance can be transferred from *L. reuteri* to *E. faecium* and from *E. faecium* to *E. faecalis* in the mouse gastrointestinal tract.[17]

A study by Temmerman *et al.*,[9] found 68.4% of probiotic isolates were resistant to two or more antibiotics. Strains of lactobacilli were found resistant to kanamycin (81%), tetracycline (29.5%), erythromycin (12%) and chloramphenicol (8.5%). The disc diffusion method showed 38% of *E. faecium* isolates were resistant to vancomycin, while the PCR based *van* gene detection assay showed they were susceptible.

Salminen *et al.*,[18] characterised 86 clinical *Lactobacillus* blood isolates at species level and tested them for antimicrobial sensitivity. Of the eleven species identified 46 isolates were *L. rhamnosus* (n=22 *L. rhamnosus* GG type), *Lactobacillus fermentum* (n=12) and *Lactobacillus casei* (n=12). All *Lactobacillus* isolates showed low minimum inhibitory concentrations (MICs) of imipenem, piperacillin-tazobactam, erythromycin and clindamycin. The range of MICs of cephalosporin varied widely with species while MICs of vancomycin were high except for *Lactobacillus gasseri* and *Lactobacillus jensenii*. The antimicrobial susceptibility pattern for probiotic *L. rhamnosus* GG was similar to those of *L. rhamnosus* GG type and other *L. rhamnosus* clinical isolates. This study of a large number of blood culture isolates of *Lactobacillus* indicates their antimicrobial sensitivity to be species dependent.

Sullivan and Nord[19] characterised the *Lactobacillus* blood isolates from bacteraemic patients in Stockholm, Sweden, between January 1998 and March 2004 to identify the possible presence of three probiotic strains of *Lactobacillus* consumed in Sweden. The majority of the 59 isolates were *L. rhamnosus* (n=17), *L. paracasei ssp. paracasei* (n=8) and *L. plantarum* (n=8). No isolates were identical to the probiotic strains. All isolates of *L. rhamnosus, L. paracasei ssp. paracasei* and *L. plantarum* were resistant to vancomycin and teicoplanin while the majority of isolates were susceptible to clindamycin.

The potential for gene transfer is difficult to assess *in vivo*. It is also difficult to assess what level of gene transfer, if any, the community may consider acceptable. It is a significant reason to select strains lacking the potential to transfer genetic determinants of antibiotic

resistance. There is little basis for scientific regulation of strains with intrinsic resistance, as little is known about the levels of intrinsic resistance in current probiotic and food strains. Systematic screening for antibiotic resistance in probiotic strains is not undertaken at present. It is essential that probiotic organisms be sensitive to broad spectrum and commonly used antibiotics.

Immune modulation

The gut microflora are the major source of microbial stimulus in infancy. The initial colonisation by and composition of the gut microflora are pivotal to the development of immune responses and normal gut barrier function. Kalliomäki et al.,[20] demonstrated that the composition of gut microflora differs between healthy and allergic infants. In a standardized double-blind placebo-controlled trial L. rhamnosus GG was given to mothers prenatally for two weeks before delivery and six months postnatally if breast feeding, or to the infant if not. No adverse effects were observed in the mothers and in infants the incidence of atopic eczema in the first two years of life was halved compared to that in infants given placebo.

The finding that a specific strain of probiotic bacteria strongly influences immune regulation in infants raises questions about the use of probiotics in infancy. The long-term effects of probiotics on the composition of the gut flora and gut immunity during maturation are unknown. Reid[21] questions that probiotic safety be assessed solely by an absence of adverse effects, and proposes longer term endpoints to determine whether there is increased risk of incurring diseases such as diabetes and inflammatory disorders.

Once a probiotic strain is incorporated into the normal microflora, as has been documented during infancy, the potential to stimulate an immune response may be abolished with a consequent loss of probiotic potential. The response of normal gut microflora to probiotic intervention varies with age and clinical status of the subject, so immunological effects need to be assessed in specific at-risk populations. The molecular factors modulating immunoregulation need to be elucidated. Safety evaluation of long-term health effects will be important in the selection of, and characterisation studies for a probiotic.

Clinical studies

Clinical studies in humans have investigated the effect of oral administration of probiotics on the balance of intestinal microflora and in a variety of disorders. Until recently many studies were of inadequate design and produced unreliable data. Inadequate studies have had an absence of a patient control group; small treatment groups; undefined treatment groups; a wide age range within a treatment group; a diversity of antibiotic treatments; an absence of dosing criteria such as dose and duration; or subjects with symptoms of concurrent disease with the potential to confound an observation of adverse effects. The gold standard is a controlled study with standardized, blind assignation to treatment, placebo and untreated groups.

Immunosuppressive therapy is considered a risk factor in bacteraemia from opportunistic pathogens. Salminen et al.,[22] evaluated the efficacy and safety of Lactobacillus rhamnosus GG (LGG) in moderating gastrointestinal symptoms of HIV-positive patients on antiretroviral therapy in a placebo-controlled double blinded crossover study. Subjects with HIV infection and persistent non-infectious diarrhoea taking highly active antiretroviral therapy were standardized to receive twice daily LGG (viable LGG 1-5 x 10^{10} cfu/dose) for two weeks and two weeks placebo in randomized order. No probiotic products were permitted during the washout periods before and after each treatment, to reduce the likelihood of a carryover effect from persistent probiotic. Although the LGG preparation was well tolerated it gave no significant reduction in gastrointestinal symptoms. No adverse events or clinical infections were observed in the subjects during the study or in the six month follow-up period. The evidence from this study suggests that LGG is unlikely to be a health risk in HIV patients.

Epidemiological surveillance

Two Finnish studies have investigated the incidence of infections associated with lactic acid bacteria. In the first study 16S rRNA methods were used to characterise and identify lactic acid bacteria isolated from blood cultures of bacteraemic patients in Southern Finland.[23] The number of infections caused by lactobacilli was extremely low and the infections were not associated with the probiotic strain newly introduced in fermented milks.

In a subsequent study, lactobacilli isolated from bacteraemic patients between 1989 and 1994 were compared to common dairy or pharmaceutical strains.[24] From a total of 5192 blood cultures 12 were positive for lactobacilli, an incidence of 0.23%. None of the clinical cases could be related to lactobacilli strains used by the dairy industry. In both studies, patients with lactic acid bacteria bacteraemia had other severe underlying illnesses.

Salminen et al.,[25] examined the incidence of lactobacilli bacteraemia in the Finnish population for the period corresponding to a rapid increase in consumption of the probiotic strain Lactobacillus rhamnosus GG (ATCC 53103). This strain was isolated from human intestinal flora and introduced into dairy products in 1990. By 1999 the annual per capita consumption was estimated at 6L (3 x 10^{11}cfu) per person/year.

The Helsinki University Central Hospital collected all Lactobacillus isolates from blood cultures and cerebrospinal fluid in its catchment area from 1990 to 2000. Blood culture isolates were also collected for all cases of Lactobacillus bacteraemia reported (and unreported) by mandatory notification to the National Infectious Disease Register, from its inception in 1995 to 2000. Species were characterised and compared to L. rhamnosus GG strain by molecular epidemiological methods.

Ninety cases of Lactobacillus bacteraemia were identified between 1995 and 2000, when the population in Finland was 5.2 million. Of the 66 isolates available for species-level identification 48 were Lactobacillus isolates, with the most common species being L. rhamnosus (26, 54%), L. fermentum (9, 19%) and L. casei (7, 15%) respectively. In 35 cases more than one additional bacterial species other then Lactobacillus was also identified. Eighteen of the 66 isolates (27%) were organisms

other than *Lactobacillus*. Eleven of the 26 *L. rhamnosus* strains were indistinguishable by PFGE from the probiotic *L. rhamnosus* GG.

No increase in the incidence or proportion of *Lactobacillus* bacteraemia was observed, despite a clear increase in the number of cases of bacteraemia over the period. *Lactobacillus* isolates as a proportion of all blood culture isolates was 0.24%, consistent with previous Finnish reports.[24] The average annual national incidence of *Lactobacillus* bacteraemia was estimated as 0.29 cases per 100,000 people per year. The study provides evidence that increased consumption of *L. rhamnosus* GG had not led to a corresponding increase in *Lactobacillus* bacteraemia.

Borriello *et al.*,[16] was unable to find published medical literature regarding the consumption of viable probiotics by hospital patients, some of whom may be predisposed to infection by probiotic bacteria. They suggested that because of the low incidence of probiotic bacteraemia and the sophisticated methods and experience needed to confirm it, identification and confirmation of species and strain characteristics of suspect clinical isolates should be referred to national reference centres. A valuable adjunct to future epidemiological studies such as that by Salminen *et al.*[25] would be an analysis of the relationship if any, between the clinical status of the patient and the presence of *Lactobacillus* bacteraemia. National clinical and epidemiological databases could include identity of organism, status of patient's underlying conditions, coexisting infections and outcomes.

All probiotics are not equal

Many bacteria are being tested to find a putative probiotic, yielding conflicting data, sometimes for the same organism. Comparisons between studies and organisms cannot be readily made because of non-standardized dosing procedures, particularly for the number of bacteria and the duration of dosing. Pharmacokinetics, pharmacodynamics, safety and the risk of acquisition of antimicrobial resistance have usually not been evaluated.[27]

Probiotic effects are strain-specific, illustrating the need to characterise the relationship between the dose, its duration and effect, on a strain by strain basis. When considering the pharmacokinetics of the probiotic organism we need to know if the bacterial strain modifies intestinal flora. In determining the dose-response relationship, if there is failure to elicit an effect is that because the organisms failed to reach effective levels at the site, or is it due to rapid elimination of the bacteria, or non-persistence, or destruction?

As far as dose is concerned, it is unclear whether proposed consumption of a probiotic is to be on a regular daily basis for whole of life, or irregular and dependent on symptoms. Information is not readily available on the equivalence or comparability of formulations in different preparations; on the distinction between spore or vegetative forms, powders, granules, tablets, liquids and yoghurts; or adult and paediatric products. Intake data are not generally available for those countries where products are used. Nutritional studies may be needed in addition to toxicological studies, depending on the nature of the product; its intended use; its anticipated intake; the impact of dietary intake on the spectrum of colonic flora, their metabolic functions and bioavailability of nutrients.[28]

Summary

It is only when a probiotic strain has been unequivocally identified; characterised, screened and its mechanisms of action elucidated with scientific rigour; labelled accurately and truthfully; tested for safe and efficacious human use in randomized, blinded placebo-controlled clinical trials, ideally with independent verification; and undergone a risk-benefit comparison with existing treatments that there will be evidence of sufficient quality to support the unjustified beneficial claims made to date for many proposed probiotics.

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Original Article

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益生菌的安全性

新品種及更多的特定的益生菌菌株，持續被當作開發新益生菌產品的對象。在將新的菌株添加到食品或是治療產品之前，必須謹慎的評估它們的功效，並評估它們是否會享有傳統食品有機體的安全性。在缺乏科學標準的情況下，經傳統的食物生產的固有食物有機體，被武斷的歸為安全的。益生菌的安全性及功效的證據，直到現在仍只是傳聞或根據相對較小且設計不良的研究。論證益生菌的效力可以為人類及獸醫產品的發展提供廣大的機會。培養一個新益生菌，至少要確定它與傳統的同類一樣安全。很多細菌曾被檢驗以找尋可能的益生菌，但是有時即使是相同的細菌也會產生矛盾的數據。因為非標準化的劑量程序，不同的研究及有機體之間無法立即互相比較。不同的益生菌製備方式間的對等公式的訊息並不可得。對於如何適當地測試一個可以被人類食用的新的益生菌菌株的安全性，引發激烈的辯論。傳統的毒物學及安全評估對評估益生菌的安全性價值有限。當要將新的細菌菌株添在食品或是治療產品中，需要重新考慮安全性評估的程序。本文獻針對這些議題提供概要論述。

關鍵字：益生菌、安全性、菌血症、臨床試驗、導引、功效。