

Urogenital Lactobacilli Probiotics, Reliability, and Regulatory Issues

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ABSTRACT

Of the hundreds of *Lactobacillus* so-called probiotic products, only a handful contain identifiable strains that have any published data supporting their usefulness in humans. Of these, *Lactobacillus rhamnosus* GR-1, *Lactobacillus fermentum* RC-14, and *Lactobacillus fermentum* B-54 possess anti-pathogen properties and colonize the intestine and vagina, conferring health benefits to women. These strains have been found to adhere to vaginal cells, hemagglutinate red blood cells and produce biosurfactants. Taking these or other strains to the marketplace, whether as dairy products or not, requires reliable production and delivery vehicles, and regulatory approval. For this important field to move forward to meet the approval of health care professionals, a better scientific basis for specific strain use in high quality products aimed at well-defined sites is needed. Furthermore, information targeted at, or viewed by, consumers should state the attributes of actual strains in a given product, and reference the peer-reviewed journal in which the work was published.

(**Key words:** probiotics, regulatory issues)

Abbreviation key: GIT = gastrointestinal tract, FOS = fructooligosaccharides.

INTRODUCTION

Dairy companies, primarily those in Japan (Yakult), Switzerland (Nestle), France (Danone), and Scandinavia (Probi, Valio) dominate the delivery of probiotic organisms to consumers. The delivery vehicles are primarily yogurt and drinks, because of the expertise of the companies and the regulatory ease with which foods can be marketed compared with pharmaceuticals. These strains, *Lactobacillus casei* Shirota, *Lactobacillus johnsonii* LJ1, *Lactobacillus casei* DN-114 001, *Lactobacillus plantarum* 299V, and *Lactobacillus rhamnosus* GG, respectively, have varying amounts of published data on their clinical attributes and mechanisms of action.

Many aspects of probiotic research and development, such as manufacturing and regulatory claims, are essential for successful commercialization of strains, yet they are rarely discussed in scientific reviews. Some of these will be discussed in this paper: namely, selection of strains and target conditions, successful upscale manufacturing, storage and shelf life, and regulatory issues. Some of the properties that

appear to be important for lactobacilli to convey host benefits, particularly in the urogenital tract of women will be discussed.

PROBIOTICS GO BEYOND THE INTESTINAL TRACT

While many criteria have been proposed as desirable for potential probiotic cultures, in practice the properties necessary are dependent on the target site towards which the probiotic is directed and the desired effect at that site. Therapeutic cultures may be directed towards a variety of ailments including lactose maldigestion, cancer, infections of the intestinal and urogenital tracts, and elevated serum cholesterol (Naidu et al., 1999). Although the gastrointestinal tract (GIT) is the principal target of available probiotic cultures—and indeed most definitions of probiotics refer to intestinal site of action—probiotic organisms have applications at other sites, such as the urogenital tract, nasopharynx, wounds, and elsewhere. Indeed, it was Sprunt and coworkers' studies of alpha-streptococci in the nasopharynx in the 1960s that laid the groundwork for some of the present interest (Sprunt and Leidy, 1988).

Factors Important for Probiotics Intended for Urogenital Tract Applications

The vagina is colonized by up to 50 species of organisms during a lifetime. In large part because of lactobacilli (which can exist at 10^7 cells/ml of vaginal fluid), this microenvironment is usually capable of protecting the host from infectious diseases, including some that are sexually transmitted or which increase the risk of preterm labor (Goldenberg et al., 1996; Redondo-Lopez et al., 1990; Sewankambo et al., 1997). Nevertheless, statistically every woman will suffer from yeast vaginitis, bacterial vaginosis, or urinary tract infection at some point in her life (Reid, 1999; Sobel, 1993, 1999).

A number of factors are believed to be important for lactobacilli to colonize the urogenital and intestinal tracts and reduce the risk of infection (Bruce and Reid, 1988; Reid, 1999; Reid et al. 1995; Sobel, 1999). Of these, adhesion to cells and mucus is believed to be one of the main factors when it coincides with survival of the organism and inhibition of growth and adhesion of pathogens. The study of adhesion with respect to the intestine has been done primarily with CaCo-2 cells, and the clinical relevance of the adhesion values has been questioned. It would require intestinal biopsies to fully correlate adhesion in vitro with those obtained in vivo. Even then, given that dense biofilms of organisms coat the mucus and cells of the intestine, the level of adhesion to cells may not be as critical as an ability to adhere to and penetrate existing biofilms. For the vagina, it is easier to obtain cells and deter-

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mine if in vitro data correlates with in vivo findings. The adhesion of *L. rhamnosus* GR-1 and *L. fermentum* B-54 to vaginal cells does somewhat correspond to in vitro counts (Reid et al. 1995), with the latter being slightly higher. Adhesins can be detected by hemagglutination reactions (essentially binding of bacteria to blood cells), and a classification system has been reported using hemagglutination to separate strains that would be potentially good probiotic agents from those that would not (Andreu et al. 1995).

Studies performed to examine the relative importance of hemagglutinins and other adhesion factors in lactobacilli strains being used as human probiotics, namely *L. rhamnosus* GR-1 and 36, and *L. fermentum* RC-14 and B-54 are outlined below.

Bacterial adhesins. When grown in broth, *L. rhamnosus* GR-1 and 36, and *L. fermentum* RC-14 and B-54 did not hemagglutinate red blood cells, compared with type 1 and P fimbriated *Escherichia coli* Hu734 (Table 1). However, growth on agar did result in expression of hemagglutinins by all four lactobacilli. The lactobacilli did not possess fimbriae when examined by transmission electron microscopy, (although some nondefinable strandlike substances were seen around the outer capsule layer of strain B-54), whereas fimbriae were abundant around *E. coli* Hu734. Therefore, the *Lactobacillus* strains tested here do not fit into any of the groupings suggested by Andreu et al. (1995), which are based upon hemagglutination patterns. All four strains would be in the 10 percentile of Andreu's strains adhering in numbers greater than 15 per cell, yet their inability to bind sheep cells excludes them from their groups II and III. None of the four strains agglutinated sheep red blood cells but each strain agglutinated type O or A and O human cells (Table 1). However, the four *Lactobacillus* strains have been found to colonize the vagina (stay at least 7 wk after its intravaginal inoculation; Reid et al. 1994). Thus, there is a danger in selecting probiotic strains based solely upon in vitro hemagglutination patterns alone, as Andreu's paper implied.

Adherence. Bacterial adherence to vaginal epithelial cells was evaluated by adding 10^5 cells/ml of vaginal epithelial cells to 10^8 cfu/ml of bacteria and incubating for 1 h at 37°C (Reid et al. 1987). Net adhesion was enumerated on 50 Gram-stained

cells by deducting control counts on cells incubated with PBS alone. All four lactobacilli adhered well to the vaginal epithelial cells as did *E. coli* Hu734 (Table 1). Strain GR-1 was the most adherent *Lactobacillus* (almost twofold greater than the others), although under the growth conditions used, it only expressed hemagglutinin reactive with O type human cells. Cell surface hydrophobicity of the strains was also measured using water contact angles (Reid et al. 1992), and did not correlate with the extent of adhesion to cells per se. The two *L. rhamnosus* strains, GR-1 and 36 with identical ribotype (Zhong et al., 1998) were found to be hydrophilic, while *L. fermentum* RC-14 and B-54 also with identical ribotype (Zhong et al., 1998) were extremely hydrophobic (Table 1). Given that strains GR-1 and B-54 have been shown to colonize the vagina and reduce the risk of infection (Reid et al. 1994, 1995), the use of cell surface hydrophobicity alone to select probiotic strains does not appear to have much merit.

Inhibition of adhesion of pathogens. Recent studies have shown that biosurfactants (compounds released by microbes with a distinct tendency to accumulate at interfaces) produced by certain lactobacilli, not only aid binding of the organisms to collagen on cells, but they inhibit adhesion to surfaces of a broad range of uropathogens (Velraeds et al., 1996, 1998; Heinemann et al. 2000; Howard et al. 2000). Biosurfactant activity was assessed by measurement of the decrease in liquid surface tension by axisymmetric drop shape analysis by profile (ADSA-P)(Reid et al. 1992b), and by the ability to inhibit adhesion of *Enterococcus faecalis* 1131 to polystyrene (Reid et al., 1999). For both of the *L. rhamnosus* strains, GR-1 and 36, the ADSA-P showed the presence of biosurfactant, while the ability to inhibit enterococci adhesion was weak (Table 1). Both *L. fermentum* RC-14 and B-54 had very active biosurfactant activity, inhibiting up to 90% adhesion of enterococci. Biosurfactant activity was shown not to correlate with presence of hemagglutinins. More recently, a 29-kDa protein (p29) that binds to collagen has been discovered within the biosurfactant mixture from RC-14, and found to inhibit enterococcal adhesion (Heinemann et al. 2000; Howard et al. 2000). Expression of p29 on vaginal cells colonized by RC-14 has now been shown (unpublished data), verifying a correlation between in vitro and in vivo data.

Importance of Strain Selection

Some of the health benefits attributed to probiotic cultures are strain dependent, stressing the importance of probiotic strain selection and highlighting the fact that claims made for one probiotic culture cannot necessarily be applied to another. For example, some commercial dairy probiotics intended for intestinal applications have been tested in our laboratory for comparative purposes. To date, results show *L. casei* Shirota, *L. rhamnosus* GG, and other commercially available strains do not possess the p29 collagen binding protein present in *L. fermentum* RC-14 (Reid and Bruce, 2001), and *L. rhamnosus* GG is significantly less able to inhibit enterococci adhesion. In addition, *L. acidophilus* NCFM did not bind as well or inhibit pathogen adhesion compared with *L. rhamnosus* GR-1 and *L. fermentum* RC-14 (Reid, 2000). These findings highlight strain differences. The inability of the GG strain to reduce the risk of UTI (Kontiohari et al. 2000) illustrates that strain differences may be important clinically. However, more research is required to understand fully why GG did not reduce UTI,

Table 1. Summary of the results for the surface and adhesion analysis of *Lactobacillus rhamnosus* GR-1, *L. rhamnosus* 36, *Lactobacillus fermentum* RC-14 and *L. fermentum* B-54 along with a control *E. coli* Hu734.

	Hemagglutination				Vaginal cell adhesion ¹	Water contact angle	Biosurfactant ²
	A	B	O	Sheep			
GR-1	-	-	+	-	25	33	±
36	-	-	±	-	16	33	±
RC-14	±	-	+	-	16	102	+
B-54	±	±	+	-	15	105	+
Hu734	++	++	++	-	36	17	-

¹Expressed as number of adherent bacteria per cell (standard deviation within 25%); - = no activity detected; ± = weak reaction; + = strong reaction; ++ very strong reaction, carried out at 4°C for 2 h.

²Biosurfactant ability to inhibit enterococcal adhesion with + at over 75% inhibition and ± at 1 to 75%.

while GR-1 with B-54 or RC-14 does (Reid et al., 1995; Reid, 2000b).

There is a need to select probiotic strains on the basis of their functional attributes, and based on this, a particular culture or mix of cultures should be chosen for certain applications. In vitro tests based on a selection criteria devised by several researchers (Collins et al. 1998; Reid, 1999b; Salminen et al. 1996; 1998; Sobel, 1999) have proved useful for assessing a bank of potential probiotic cultures; however, there is a need to correlate in vitro findings with in vivo functionality, and ultimate proof of probiotic effects requires validation in well-designed clinical trials (Guarner and Schaafsma, 1998). Furthermore, it is important that probiotic properties are retained during in vitro experimentation under laboratory conditions and also, as outlined below, during subsequent processing and storage (Conway, 1996). It is surely the responsibility of producers and distributors of probiotics to obtain the necessary conclusive evidence that their strains have any beneficial effect for the host, or any differential attributes over other strains. To date, too few companies have acquired and published this evidence. For strains that are able to colonize the urogenital tract and reduce the risk of infection, so far only GR-1, RC-14, and B-54 have any supported data (Bruce et al. 1992; Reid et al. 1992, 1995; Reid, 2000).

SUCCESSFUL UPSCALE MANUFACTURING, STORAGE, AND SHELF LIFE

The requirement for large-scale manufacture of probiotics and, thereafter, microbial survival during the relevant storage/shelf life of a product is influenced by what is considered to be the minimum effective dose for therapeutic cultures. In this respect, a daily intake of 10^6 to 10^9 viable cfu has been recommended (Lee and Salminen, 1995) but has not been validated by scientific studies, and, indeed, doses below 10^8 cfu/d should not be considered unless the delivery vehicle somehow propagates the effects in the gut. It seems that high levels of daily consumption are required as illustrated by findings that the minimum dose of *L. rhamnosus* GG required to yield fecal recovery was 10^{10} cfu/d (Saxelin et al., 1991; 1995). Furthermore, Donnet-Hughes et al. (1999) found that, although fecal recovery was found in all subjects consuming *L. johnsonii* LJ1, 10^9 cfu/d elicited immune effects, whereas a lower dose of 10^8 cfu did not. Probiotic levels recommended in food products and dietary supplements are based on these minimum therapeutic levels and are currently unclear (Hamilton-Miller and Fuller, 1996). However, based on a therapeutic minimum of 10^9 cfu/d and consumption of 100 g or ml of product, a food should contain at least 10^7 cells per gram or milliliter, which is in agreement with current Japanese recommendations (Ishibashi and Shimamura, 1993).

The issue is compounded by the fastidious nature of many lactobacilli and bifidobacterial strains used in probiotic products. Difficulties occur in scaling up their growth and retaining sufficient viable counts throughout freeze-drying and storage. For example, many probiotic cultures, especially *Bifidobacterium*, do not grow well in milk (Svensson, 1999), and this may pose problems when preparing inocula for dairy products, which are currently the most common choice for probiotic incorporation. Moreover, cultures must be able to withstand processing parameters, retain probiotic properties following

processing, and survive to high levels in the product during shelf life/storage (Knorr, 1998). Too often, products are said to contain high viable counts, when in fact most of the contents are dead or those that do survive are either not mentioned on the label or have no therapeutic use. This problem is well illustrated by a recent analysis of 52 so-called probiotic products available in Europe, where most were found to be unreliable (Hamilton-Miller et al., 1999). Another problem is bile and acid tolerance. If strains cannot survive passage through the stomach and upper intestine, they will not be present in sufficient numbers to make an impact on the colon microenvironment. Food carriers such as dairy products may enhance microbial survival in the GIT, most likely due to a buffering or protective effect. A previous study has shown superior survival upon probiotic exposure to gastric juice in Cheddar cheese compared with yogurt (Gardiner et al., 1999).

Therefore, many challenges are associated with upscaling probiotic organisms, and just because a strain shows promise in vitro or in vivo does not mean that it can be economically made available to a wider population.

Probiotic Delivery Systems

Food supplements in the form of capsules or tablets can be used for probiotic delivery, and these may have the advantage of delivering large numbers of the probiotic strains to the consumer. Indeed, several new technologies are being developed and tested that coat organisms or their carrier to help avoid stomach acid and bile salt damage.

Still, traditional dairy foods, such as freshly fermented yogurts and fermented milks, as well as unfermented milks, are a popular choice for probiotic incorporation, and provide the consumer with a tasty nutrient that can be part of his/her diet. The addition of probiotic cultures to foods can be seen as fortification with biologically active components and as such leads to the development of a "functional food" (Sanders, 1998). Few yogurts are actually made with probiotic strains as the only microbial ingredients. Indeed, starter cultures such as lactobacilli and streptococci often produce the yogurt, then probiotic organisms are added as an adjunct. The viability of the probiotic organisms is then limited to around 6 to 7 wk (thus the short sell-by date). Recently, the range of dairy products containing probiotic cultures has expanded to include cheeses, ice cream, and frozen yogurt and even nondairy foods and drinks (Gardiner et al., 1998; Stanton et al., 1998). The identification of alternative probiotic delivery systems is useful as some foods provide a more favorable milieu to support long-term probiotic survival than others; for example, Cheddar cheese is less acidic and has a considerably longer shelf life than yogurt. In addition, the delivery system may also play an important role in determining probiotic viability in the GIT following consumption. In this respect, fermented milks and enterocoated tablets were shown to be more efficacious for delivery of *L. rhamnosus* GG than a freeze-dried powder (Saxelin, 1997). Mature Cheddar cheese may offer certain advantages compared with fresh yogurt as a delivery system for viable probiotic microorganisms (Gardiner et al., 1999).

The use of freeze-dried (lyophilized) or spray-dried cultures overcomes the problems associated with preparation of probiotic cultures, particularly those that have poor growth rates in milk. A further advantage of dried cultures is their long shelf life and stability at room temperature: in Europe

Table 2. This table contains statements selected at random from various web sites by companies marketing probiotic products. The interpretation of these statements, as made in the text, is not meant to be detrimental to the products themselves as none of them have been tested by us, but rather to raise for discussion the point that products should not be advocated because other strains (not present in their product) have proven effectiveness. If these claims are real, they should be supported by scientific or clinical evidence published in peer-reviewed journals and cited on the web sites.

“Claims” or statements made on web sites

“Probiotic components gently tone, exfoliate and moisturize your skin, restoring its natural luster without the abrasiveness of alpha-hydroxy acid.”

“... is specifically designed to aid in restoring the healthy balance of vaginal and intestinal flora of women.”

“Act as ‘watchdogs’ by keeping an eye on, and effectively controlling, the spread of undesirable microorganisms.”

“... products are liquid biostimulants designed to accelerate the degradation of organic waste.”

“It’s specially selected strains of beneficial micro-organism, vitamins and minerals can be used as a preventative or as part of a programme of treatment for the following disorders:- Irritable Bowel Syndrome, Osteoporosis, Skin Problems Diarrhoea, Bloating, Flatulence, Candida Infections, M.E., Bowel Cancer, Constipation. ... is a probiotic carefully formulated for prophylactic effects in the preservation and restoration of a healthy intestinal flora in children and infants.”

“... is extremely aggressive against pathological molds, yeast, fungi and viruses.”

“... is a synergistic combination of: Acidophilus/Bifidum DDS - 1 (intestinal flora complex): Friendly flora, Lactobacilli and B. bifidum, essential for a balanced intestinal ecosystem.”

“Homeostatic Soil Organisms colonize in the colon, burrow behind putrefaction and dislodge it, and restore bacterial balance by crowding out the “bad” bacteria and lowering colonic pH.”

“... caplets support the body’s ability to digest food and nutrition at the gastrointestinal level while also providing natural fibers necessary for proper colon function.”

this is tested at 20°C, while in the United States the standard is set at 25°C. Spray drying, one of the principal processes used in the dairy industry, provides a relatively inexpensive method of large-scale culture production (Knorr, 1998). A recent study demonstrates that spray drying offers potential as a cost-effective means of producing large quantities of some probiotic lactobacilli in a form suitable for storage, transport, and subsequent addition to food or pharmaceutical products (Gardiner et al., 2000). In fact, the use of spray-dried powders as probiotic delivery systems overcomes the problem of restricted shelf life/storage associated with certain dairy products, in particular yogurts and fermented milks.

REGULATORY APPROVAL

Government regulations differ in every country, but for the most part probiotics come under foods and dietary supplements because most are delivered by mouth as foods (thus the term functional foods) or in capsules sold in health food stores and via the Internet. However, as probiotics are being applied to other areas (wounds, skin, urogenital tract, etc.), such regulations need to be revisited. Most companies get around legislation (which accounts for claims on labels and packages) by using web sites, brochures, and other means to associate their products with healthy benefits. Thus, the consumer can be led to believe that a particular probiotic product, or by association all probiotic products, cure yeast infections, treat irritable bowel syndrome, etc. (Table 2). This is a big problem as consumers rarely have the time or tools to determine whether a particular product has scientific proof of efficacy, or to know whether a given product contains an effective number of viable organisms.

It is unfortunate that too many probiotic products exist that contain strains of dubious merit and dubious viability. Companies adhere to labeling regulations but come close to violating public trust in other publicity tools, such as web sites that tell half truths (Table 2). These companies need to vali-

date the organisms they sell, publish in peer-reviewed journals the organisms’ mechanisms of action using rigorous in vitro and in vivo testing, and not market products based upon an association with other well studied strains. Not all strains of *L. acidophilus* are alike, and this is true for *L. rhamnosus*, *L. casei*, *L. reuteri*, *L. fermentum*, and other species. Scientific evidence is needed to prove that lactobacilli restore a skin’s “natural luster,” “act as watchdogs,” “accelerate the degradation of organic waste,” prevent or treat “osteoporosis,” act “aggressively against pathological molds,” or “burrow behind putrefaction.” Without this evidence, it will be difficult for professional caregivers, especially academic physicians to believe that probiotics have merit.

The same can be said for prebiotics (nondigestible dietary supplements that stimulate the growth and activity of beneficial organisms) such as fructo-oligosaccharides (FOS), lactulose, lactitol and inulin (Crittenden, 1999; Gibson and Roberfroid, 1995). The concept has some potential benefits. By increasing the metabolic activity of the “protective/beneficial” bacteria, these nutrients could help promote health in the host. However, large amounts of FOS need to be ingested to obtain an effect. In one study, 15g/d of FOS for 15 d was shown to increase the total fecal bifidobacterial content (Gibson et al. 1995). Thus, simply having FOS as a constituent of a dietary supplement is insufficient to confer host benefits, and such claims should not be made on product literature or web sites.

There are exceptions. Valio of Finland and Yakult of Japan, for example, have web sites that contain reference to peer-reviewed publications showing some good evidence of efficacy that *L. rhamnosus* GG and *L. casei* Shirota, respectively, can be effective, for example in preventing and reducing the duration of diarrhea. While few consumers might actually read these manuscripts, their existence adds credibility and a point of reference for people wanting to find out more about certain product lines.

CONCLUSIONS

Marketing information shows that North America is prime for probiotic products, and globally there is a need for such natural remedies to prevent disease and restore and maintain health (Brower, 1999; Childs, 1997; Reid, 2000b; Stanton et al., 2000). There seems little doubt that probiotics will become part of a global health supplement approach. The question is, will properly tested products be used, long-term follow-up studies be published, and scientific investigations into mechanisms of action be funded? Time will tell.

Critics point to antibiotics as “gold standards” in management of disease, even as prophylactic agents to prevent recurrences, such as in urinary tract infections. This is quite amazing when one considers the enormous side effects (evident in the Compendium of Pharmaceuticals and Specialties manual of drugs) associated with antibiotics, including hypersensitivity, induction of yeast vaginitis, and death. On the contrary, probiotic applications have been relatively devoid of side effects (Naidu et al. 1999). Hopefully, when the FDA considers approval of probiotic products, it will recognize that approved antibiotics have significantly more deficiencies in terms of side effects than probiotic organisms.

Great strides are being made in understanding the normal flora of the skin, intestine, and urogenital tract, but we don't yet understand the parameters of a normal healthy balance. As Tannock and colleagues (2000) have shown, the intestinal flora is highly complex and contains many organisms that are clearly with us for life. To dislodge, replace, or integrate into that flora is not an easy task, and certainly not one that will be achieved by each and every *Lactobacillus* strain. Only by understanding what goes on inside this milieu, will we be better placed to deliver modifiers including probiotic organisms and prebiotics that confer health benefits to the host. A handful of such strains exist as first line agents for this purpose. In time, scientific breakthroughs will provide second, third, and fourth line products with even greater health benefits, perhaps starting immediately upon birth. Such breakthroughs can only occur if further studies are sponsored, peer-reviewed, and promoted.

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