In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings\textsuperscript{1–4}

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ABSTRACT The enteric flora comprises \( \approx 95\% \) of the total number of cells in the human body and can elicit immune responses while protecting against microbial pathogens. However, the resident bacterial flora of the gastrointestinal tract may also be implicated in the pathogenesis of diseases such as inflammatory bowel disease (ulcerative colitis and Crohn disease). The objectives of the Probiotic Research Group based at University College Cork were to isolate and identify lactic acid bacteria exhibiting beneficial probiotic traits, such as bile tolerance in the absence of deconjugation activity, acid resistance, adherence to host epithelial tissue, and in vitro antagonism of pathogenic microorganisms or those suspected of promoting inflammation. To isolate potentially effective probiotic bacteria, we screened the microbial population adhering to surgically resected segments of the gastrointestinal tract (the environment in which they may subsequently be reintroduced and required to function). In total, 1500 bacterial strains from resected human terminal ilea were assessed. From among these organisms, \textit{Lactobacillus salivarius} subsp. \textit{salivarius} strain UCC118 was selected for further study. In mouse feeding trials, milk-borne \textit{L. salivarius} strain UCC118 could successfully colonize the murine gastrointestinal tract. A human feeding study conducted in 80 healthy volunteers showed that yogurt can be used as a vehicle for delivery of strain UCC118 to the human gastrointestinal tract with considerable efficacy in influencing gut flora and colonization. In summary, we developed criteria for in vitro selection of probiotic bacteria that may reflect certain in vivo effects on the host such as modulation of gastrointestinal tract microflora. 

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KEY WORDS Probiotics, lactic acid, bacteria, isolation, assessment, feeding studies, mouse studies, human studies, selection criteria

INTRODUCTION

As today’s consumers become increasingly aware of the processes that may be necessary for maintenance of their environment, health, and nutrition, scientific research has focused on the roles that diet, stress, and modern medical practices (eg, the use of antibiotics and radiotherapy) play in threatening human health. In particular, the shifting of population dynamics toward older societies is increasing the incidence of illnesses that may be caused by deficient or compromised microflora, such as gastrointestinal tract infections, constipation, irritable bowel syndrome, inflammatory bowel disease (Crohn disease and ulcerative colitis), food allergies, antibiotic-induced diarrhea, cardiovascular disease, and certain cancers (eg, colorectal cancer) (1–3). Furthermore, concern has been expressed as the degree of microbial resistance to indiscriminately prescribed and misused antibiotics increases. To combat these trends directly, the World Health Organization currently advocates the implementation of alternative disease control strategies, such as exploiting the prophylactic and therapeutic potential of probiotic bacteria (4, 5).

Probiotics have been defined as “a live microbial food supplement which beneficially affects the host by improving the intestinal microbial balance” (6) and, more broadly, as “living microorganisms, which upon ingestion in certain numbers, exert health affects beyond inherent basic nutrition” (7). Cocktails of various microorganisms, particularly species of \textit{Lactobacillus} and \textit{Streptococcus}, have traditionally been used in fermented dairy products to promote human health. However, it was Metchnikoff in 1907 who first implied that ingested bacteria, in the form of yogurt and other fermented foods, could beneficially affect the normal gut flora (8). Numerous studies from subsequent research programs reported the generally unsubstantiated health-promoting properties of lactic acid bacteria, yeast, and fermented dairy products in animals and humans. These properties include the beneficial influences probiotics apparently exert on the microbial ecology of the host, lactose intolerance, incidence of diarrhea, mucosal immune response, blood cholesterol concentrations, and cancer (2).

In recent years, the commercial manufacture and marketing of functional foods (foods that affect functions of the body in a targeted manner so as to bring about positive effects on physiology and nutrition), particularly probiotic (acidophilus-bifidus)
yogurts, has spread from the well-established Japanese niche marketplace into the lucrative and expanding European Union marketplace. Although several probiotic bacteria of human origin are now being exploited commercially [e.g., Lactobacillus rhamnosus GG (9), Lactobacillus casei Shirota (10, 11), and Lactobacillus acidophilus LA-1 (12)], many consumers, consumer organizations, and members of the scientific community are skeptical of such products and their publicized probiotic claims (13). The dairy food industry is therefore under considerable pressure to scientifically validate these and new probiotic food products. Previously, efforts to derive complementary scientific evidence suffered because of difficulties in definitively identifying the bacterial strains involved, differences in experimental procedures, subjective data interpretation, and poor communication between academic scientists, the dairy food industry, and clinicians (4, 14). However, workshops similar to those hosted by European Union–funded programs such as The Lactic Acid Bacteria Industrial Platform (LABIP) and PROBDEMO (FAIR CT-96 1028) (15) promote the dissemination of scientific results, the generation of consensus, and the direct interaction of research institutions and universities with industry.

In 1998 the participants involved in the LABIP workshop on probiotics concluded that “probiotics may be consumed either as a food component or as a non-food preparation” and supported criteria outlined by others for the selection and assessment of probiotic lactic acid bacteria (7). These criteria may be summarized as follows: human origin, nonpathogenic behavior, resistance to technological processes (i.e., viability and activity in delivery vehicles), resistance to gastric acidity and bile toxicity, adhesion to gut epithelial tissue, ability to persist within the gastrointestinal tract, production of antimicrobial substances, ability to modulate immune responses, and ability to influence metabolic activities (e.g., cholesterol assimilation, lactase activity, and vitamin production) (7, 16–20). The participants of the LABIP workshop on probiotics also concluded that the demonstration of probiotic activity of a certain strain requires well-designed, double-blind, placebo-controlled human studies (7). However, these requirements were further outlined by Berg (21) and Salminen et al (2, 20) as follows: each potential probiotic strain should be documented and assessed independently; extrapolation of data from closely related strains is not acceptable; only well-defined strains, products, and study populations should be used in trials; when possible, all human studies should be randomized, double-blind, and placebo-controlled; results should be confirmed by independent research groups; and preferentially, the study should be published in a peer-reviewed journal.

### ISOLATION OF POTENTIAL PROBIOTIC BACTERIAL STRAINS

Currently, probiotics are being used successfully to improve the quality of feed provided to domestic animals (22). The resulting benefits of effective administration of probiotics in feed to cattle, pigs, and chickens include enhanced general health, faster growth rates as a result of improved nutrition, and increased production of milk and eggs (21). Some of the microorganisms most commonly used to promote animal health and nutrition include strains of the Lactobacillus, Bifidobacterium, Bacillus, Streptococcus, Pediococcus, Enterococcus genera and yeast of the Saccharomyces, Aspergillus, and Torulopsis genera (18). In the development of probiotic foods intended for human consumption, strains of lactic acid bacteria, such as Lactobacillus, Bifidobacterium, and Streptococcus, have been used most commonly, primarily because of the perception that they are desirable members of the intestinal microflora (21, 23) (Table 1). In addition, these bacteria have traditionally been used in the manufacture of fermented dairy products and have GRAS (generally regarded as safe) status (34). However, some of the probiotic isolates currently used in the dairy food industry are not of human origin and therefore do not meet the criteria outlined above for the selection of acceptable probiotic microorganisms.

At University College Cork, the Probiotic Research Group recognized that lactobacilli isolated from human feces have been relatively well characterized (35). However, it was further recognized that few attempts had been made to isolate potential adherent probiotic bacteria directly from the human intestinal mucosa, the environment in which they may subsequently be reintroduced and required to function. The enteric flora, including >500 bacterial species, comprises ~95% of the total number of cells in the human body and contributes significantly to the host’s resistance to infectious disease. Furthermore, changes in the composition of the intestinal flora are often associated with disease and may, in some cases, be the cause of disease (36). In addition, although bifidobacteria and lactobacilli are the dominant bacterial species

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**TABLE 1**

Some probiotic bacterial and yeast strains and their reported effects

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reported effects in clinical studies</th>
<th>Selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus acidophilus</em> LC1</td>
<td>Immune enhancing, vaccine adjuvant, adherence to human intestinal cells, balancing of intestinal microflora</td>
<td>(12)</td>
</tr>
<tr>
<td><em>L. acidophilus</em> NCF01748</td>
<td>Lowering of fecal enzymes, prevention of radiotherapy-related diarrhea, treatment of constipation</td>
<td>(24, 25)</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> GG</td>
<td>Prevention of antibiotic-associated diarrhea, treatment and prevention of rotavirus diarrhea, treatment of relapsing <em>Clostridium difficile</em> diarrhea, prevention of acute diarrhea, alleviation of Crohn disease, antagonistic against carcinogenic bacteria</td>
<td>(24, 26–29)</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em> Shirota</td>
<td>Prevention of intestinal disturbances, balancing of intestinal bacteria, lowering of fecal enzymes, inhibition of superficial bladder cancer</td>
<td>(10, 11)</td>
</tr>
<tr>
<td><em>Lactobacillus gasseri</em></td>
<td>Fecal enzyme reduction, survival in the intestinal tract</td>
<td>(30)</td>
</tr>
<tr>
<td><em>Bifidobacterium bifidum</em></td>
<td>Treatment of rotavirus diarrhea, balancing of intestinal microflora, treatment of viral diarrhea</td>
<td>(31)</td>
</tr>
<tr>
<td><em>Saccharomyces boulardii</em></td>
<td>Prevention of traveler’s diarrhea, prevention and treatment of <em>C. difficile</em> diarrhea</td>
<td>(32)</td>
</tr>
</tbody>
</table>

1 Adapted from Lee and Salminen (33).
found in the feces of breast-fed infants (37), controversy still exists as to which species dominate in the gastrointestinal tract. Although *L. acidophilus* has been recovered in relatively high numbers from the gastrointestinal tract, more reports have shown that other *Lactobacillus* groups (*Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus salivarius, and Lactobacillus reuteri*) can also be isolated in similar amounts (38, 39), indicating the lack of a single dominant species.

The human gastrointestinal tract can be divided into 4 anatomical regions: the esophagus, the stomach, the small intestine (consisting of the duodenum, jejunum, and ileum), and the large intestine or colon. At University College Cork, bacteria were isolated from resected human terminal ilea obtained from patients undergoing urinary tract reconstructive surgery. After the luminal contents were initially washed out with one-quarter strength Ringer solution, the samples obtained and subjected to increasingly vigorous shaking in cysteinated one-quarter strength Ringer solution (Merck KgaA, Darmstadt, Germany) and subjected to increasingly vigorous shaking in cysteinated one-quarter strength Ringer solution, the samples obtained from 7 patients were found to contain between 1 × 10^2 and 1 × 10^3 colony-forming units (CFU)/g tissue when incubated anaerobically at 37°C for 2–5 d in a variety of media (40). Approximately 1500 catalase-negative bacterial isolates were chosen and characterized further. Of these, >60% were gram-positive homofermentative cocci, ~18% were gram-negative rods and heterofermentative coccobacilli, and 22% were predominantly homofermentative coccobacilli (40). Of the last group, 38 gram-positive isolates were further characterized by physiologic and biochemical means. Of these microorganisms, 28 belonged to the *Lactobacillus* genus (mainly *Lactobacillus paracasei* and *L. salivarius*) (40).

In addition to lactobacilli, presumptive strains of *Bifidobacterium* were isolated from homogenized ileal tissue samples. With use of molecular analysis, these bifidobacteria were classified as *Bifidobacterium infantis* (41). Interestingly, these results differ significantly from previous observations that adults do not possess detectable amounts of *B. infantis* but rather *Bifidobacterium longum* and *Bifidobacterium bifidum* (38).

### IN VITRO ASSESSMENT OF POTENTIAL PROBIOTIC BACTERIAL STRAINS

As stated earlier, several research groups have recommended that the assessment of potential probiotics involve assessment of resistance to gastric acidity and bile toxicity, adhesion to gut epithelial tissue, ability to colonize the gastrointestinal tract, production of antimicrobial substances, and ability to modulate immune responses (7, 16–20, 42). Therefore, a subset of the bacterial strains we isolated from surgically removed samples of human ilea were evaluated within these parameters under laboratory conditions.

### Resistance to gastric acidity

Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach (43). There, the secretion of gastric acid constitutes a primary defense mechanism against most ingested microorganisms. In fact, gastric surgery or the administration of acid blockers such as proton pump inhibitors may permit microbial colonization of the stomach (44). Therefore, preliminary experiments were completed to determine the degree of acid resistance exhibited by several *Lactobacillus* and *Bifidobacterium* strains isolated from the human ileum. These results were then compared directly with the resistance of several additional *Lactobacillus* strains obtained from independent research groups (Table 2). Survival of the bacterial strains was initially assessed by adding ~1 × 10^11 and 1 × 10^9 CFU lactobacilli and bifidobacteria/L, respectively, to MRS medium (45) amended with hydrogen chloride to pH values of between 2.0 and 3.4. However, survival of bacterial strains in human gastric juice is a more accurate indication of the ability of strains to survive passage through the stomach. For this reason, human gastric juice was obtained from healthy subjects by aspiration through a nasogastric tube. Because the pH of the stomach is known to fluctuate [eg, when an individual is fasting the stomach pH may be as low as 1.5 (46)], the pH of the obtained samples was Quantified before use. The gastric juice was then added to MRS medium as described above. Analysis of the results showed that many of

### Survival of some strains of *Lactobacillus* and *Bifidobacterium* in human gastric juice at different pH values

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>pH</th>
<th>0</th>
<th>5</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
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<td><em>L. casei</em> 161</td>
<td>UCC</td>
<td>1.2</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
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<td>9250</td>
<td>9290</td>
<td>8550</td>
<td>8510</td>
</tr>
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<td>Arla</td>
<td>1.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
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<td>8350</td>
<td>8170</td>
<td>7090</td>
<td>6290</td>
</tr>
<tr>
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<td>Arla</td>
<td>1.2</td>
<td>8740</td>
<td>4900</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>8440</td>
<td>8460</td>
<td>8410</td>
<td>8390</td>
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<td>9640</td>
<td>0</td>
<td>ND</td>
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<tr>
<td></td>
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<td>8600</td>
<td>8620</td>
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<td>9090</td>
</tr>
<tr>
<td><em>L. paracasei</em> 212.3</td>
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<td>1.2</td>
<td>9030</td>
<td>3370</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
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<td>8600</td>
<td>8610</td>
<td>8520</td>
<td>8600</td>
</tr>
<tr>
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<td>AUA</td>
<td>1.2</td>
<td>9090</td>
<td>9070</td>
<td>4330</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>8470</td>
<td>8400</td>
<td>8450</td>
<td>8420</td>
</tr>
<tr>
<td><em>L. salivarius</em> UCC118</td>
<td>Present studies</td>
<td>1.2</td>
<td>9000</td>
<td>8930</td>
<td>4240</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>9570</td>
<td>9580</td>
<td>9620</td>
<td>9550</td>
</tr>
<tr>
<td><em>Bifidobacterium</em> sp. 35658</td>
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<td>0</td>
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<td></td>
<td></td>
<td>2.5</td>
<td>7890</td>
<td>6450</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1. ND, not determined.

2. Sources are as follows: UCC, University College Cork, Cork, Ireland; Arla, Stockholm, Sweden; and AUA, Agricultural University of Athens, Athens, Greece.
Although the tested Escherichia coli conjugated bile acids exhibit antibacterial activity inhibiting the result of microbial activity (50–52). Both conjugated and deconjugated bile acids (deconjugation, dehydroxylation, dehydroacetate) their ability to resist the effects of bile acids (33). Bile acids effective probiotics it is generally considered necessary to evaluate when exposed to human gastric juice (Table 2).

In these preliminary studies, the objective of the Probiotic Research Group was to determine the level of acid resistance exhibited by several of the Lactobacillus and Bifidobacterium strains isolated from the human ileum. In a manner analogous to that described above, solid media were supplemented with bovine bile (Sigma Chemical Co Ltd, Poole, United Kingdom), porcine bile (Sigma), and human bile (obtained by laparoscopic cholecystectomy) to final concentrations of between 0.3% and 7.5%. These plates were then incubated at 37°C under anaerobic conditions and growth was recorded after 24–48 h (Table 3). Although the tested Lactobacillus and Bifidobacterium strains exhibited resistance to the bovine bile used, the porcine bile used in these assays proved to be significantly more inhibitory to both of the bacterial groups. However, in relation to the assessment of probiotic strains intended for human consumption, the most relevant determination is that of the strains’ ability to grow in human bile. Interestingly, regardless of the resistance patterns observed in the presence of either bovine or porcine bile, all of the assayed bacteria could grow in physiologically relevant concentrations of human bile (40).

Adherence of ileal bacterial isolates to human cell lines

The participants involved in the LABIP workshop on probiotics concluded that adhesion to gut epithelial tissue and the ability to colonize the gastrointestinal tract should be assessed during preselection of probiotic bacterial strains (7). The relative importance of these traits is further highlighted by the fact that many probiotics do not colonize their targeted hosts. Indeed, of those currently available, only L. rhamnosus GG remains within the gastrointestinal tract for any significant period of time (21, 23).

HT-29 and Caco-2 cells are human intestinal cell lines expressing morphologic and physiologic characteristics of normal human enterocytes (16) that have been exploited to elucidate the mechanisms mediating enteropathogen adhesion (12, 57–60). In more recent studies, however, these cell lines were used to select for and subsequently assess lactic acid bacteria on the basis of their adherence properties (61–66). In our studies, completed by using both HT-29 and Caco-2 cell lines, the observed adherence of Lactobacillus strains compared well with that of the well-characterized adherent L. rhamnosus GG strain (40). However, only low degrees of adherence were observed with the Bifidobacterium strains, regardless of the cell line used (40).

Antimicrobial activity

Several metabolic compounds produced by lactic acid bacteria (including organic acids, fatty acids, hydrogen peroxide, and diacetyl) have antimicrobial effects (67). However, bacteriocins or proteinaceous substances with specific inhibitory activity against closely related species are perhaps the most extensively studied (67–69). Of these, nisin, which is produced by some Lactobacillus lactis subsp. lactis strains is at present the only purified bacteriocin approved for use in products intended for human consumption (70, 71). In our studies, the lactobacilli and bifidobacteria isolated from the human ileum were assayed for antimicrobial activity against a range of indicator bacteria, including strains of Listeria, Bacillus, Enterococcus, Staphylococcus, Clostridium, Pseudomonas, E. coli, Lactobacillus, Streptococcus, Bifidobacterium, and Lactococcus. L. salivarius UCC118 was found to perform consistently well against several of the indicator strains (40). Notably, however, neither lactobacilli (with the exception of Lactobacillus fermentum) nor bifidobacteria were inhibited by strain UCC118 (40).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source2</th>
<th>Bovine bile</th>
<th>Porcine bile</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. casei 161</td>
<td>UCC</td>
<td>5.0</td>
<td>0.0</td>
</tr>
<tr>
<td>L. acidophilus 1748</td>
<td>Arla</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>L. casei F19</td>
<td>Arla</td>
<td>ND</td>
<td>0.0</td>
</tr>
<tr>
<td>L. fermentum KLD</td>
<td>Arla</td>
<td>ND</td>
<td>0.0</td>
</tr>
<tr>
<td>L. paracasei 212.3</td>
<td>AUA</td>
<td>7.5</td>
<td>0.0</td>
</tr>
<tr>
<td>L. acidophilus 242</td>
<td>AUA</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>L. salivarius UCC118</td>
<td>Present studies</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Bifidobacterium sp. 35658</td>
<td>Present studies</td>
<td>1.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

1ND, not determined.

2Sources are as follows: UCC, University College Cork, Cork, Ireland; Arla, Stockholm, Sweden; and AUA, Agricultural University of Athens, Athens, Greece.

IN VIVO ASSESSMENT OF POTENTIAL PROBIOTIC BACTERIAL STRAINS: MOUSE AND HUMAN FEEDING TRIALS

To date, the benefits of probiotics have predominantly been shown under defined and well-controlled laboratory conditions. In response, the LABIP workshop participants, among others, stated that although several in vitro assays or animal studies...are very useful in the preselection of (probiotic) bacterial strains...the proof of efficacy in humans should be granted by at least one well-designed human study” (7, 21, 42). Several prospective studies showed the efficacy of administration of lactic acid bacteria for both prophylactic and therapeutic use against diarrhea in premature infants (72), newborns (73), children (74),...
and the elderly (75) and in the therapy of antibiotic-related diarrhea (76) and traveler’s diarrhea (77).

As described above, the criteria used in our studies to select and assess potential probiotic bacterial strains resulted in the identification of a small number of Lactobacillus and Bifidobacterium strains capable of resisting the effects of bile and low pH, adhering to human epithelial cell lines, and exhibiting antimicrobial activity in vitro (40). The ability of several of these bacterial isolates to survive transit through the gastrointestinal tract was then assessed. Initially, these evaluations were completed in mice (78), focusing on 1) whether we could effectively deliver the probiotic microorganism to the gastrointestinal tract; 2) whether the introduced strains would survive transit through, and possibly colonize, the murine gastrointestinal tract; and 3) given the complexity of the hostile gastrointestinal tract and fecal environments, whether we could develop a method of counting the introduced bacterial strains by use of molecular techniques (such as those described in references 79–83) or conventional microbiological techniques. In summary, the results of this study showed that it was possible to enumerate an antibiotic-resistant variant of L. salivarius strain UCC118 from collected feces after daily administration and subsequent transit through the murine gastrointestinal tract (78).

A subsequent double-blind, placebo-controlled, ethically approved feeding trial in 80 volunteers was conducted to compare the efficacy of 2 standard food systems (fermented milk and fresh milk) in delivering L. salivarius strain UCC118 to the human gastrointestinal tract (84, 85). The results of this trial indicated that both fresh milk and yogurt are effective vehicles for the bacterial strain (84). In addition, in a proportion of volunteers (<10%), significant numbers of strain UCC118 were still detectable in feces 3 wk after cessation of its administration, indicating that the strain was capable of persisting, albeit for a short period, within the human gastrointestinal tract (84).

CONCLUSIONS

In these studies, we showed that the adoption of logical criteria for the in vitro selection of probiotic bacteria can result in the isolation of strains capable of performing effectively in the gastrointestinal tract. However, given that the human gastrointestinal tract is a complex and hostile environment, it appears unlikely that a single probiotic bacterial strain will be capable of influencing the microbial ecology of the host and of beneficially affecting lactose intolerance, the incidence of diarrhea, mucosal immune responses, blood cholesterol concentrations, and the induction of cancer. It is more likely that these effects will require the introduction of a consortium of strains, such as those observed in our studies (eg, lactobacilli and bifidobacteria). Furthermore, it is essential that these probiotic strains not be developed as individual entities but rather as the active ingredients of the food products that are ultimately intended for human consumption.

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