Identification of bacterial strains in seven probiotic products using 16S DNA sequencing

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Abstract

Many viable gut-associated bacterial strains are marketed for use as probiotic products for the treatment of gastrointestinal ailments. The most common components of probiotic formulations are species of *lactobacilli* and *bifidobacteria*. These bacteria have been shown to augment the immune system, shorten infections and mediate allergic reactions. The purpose of this study was to use 16S DNA sequencing to identify viable strains found in seven probiotic products and compare them to the manufacturers’ labels. Six of the seven probiotics contained viable bacteria, but none of those products contained viable cultures of all of the species on their labels. Two species identified were not included on their product labels. This study showed the importance of quality control when dealing with living organisms meant for human consumption. Products that are not properly labeled hinder the ability to prove the efficacy of probiotics regarding their purported claims.

Introduction

The health of the host is often dependent on the presence of a normal mucosa-associated microbiota. Bacterial strains, including various species of *bifidobacteria* and *lactobacilli*, naturally associated with these surfaces have demonstrated antagonistic activity against pathogenic bacteria both *in vitro* and *in vivo* (Hudault *et al.* 1997; Tsai *et al.* 2005). Many viable cultures of *bifidobacteria* and *lactobacilli* species are available for purchase under the label of “probiotics,” which are defined as live bacteria that confer a benefit to the host when consumed in sufficient amounts (FAO/WHO 2002). The purported benefits of probiotics include augmentation of the immune system, shortened infections, mediation of allergic reactions and facilitation of lactose digestion (Isolauri *et al.* 2001; Saavedra 2001; Van Niel *et al.* 2002).

Questions of the viability and content of probiotic products has prompted researchers worldwide to investigate manufacturers’ claims. The most common discrepancies found were the absence of species listed on the label, species misidentification, or the presence of contaminating
species (Fasoli et al. 2003; Temmerman et al. 2003; Coeuret et al. 2004; Drago et al. 2004). Contaminants, such as the yeast *Saccharomyces cerevisiae* and antibiotic resistant strains of *Enterococcus faecium*, have been identified in probiotic formulations (Hamilton-Miller et al. 1999; Temmerman et al. 2003; Elliot and Teversham 2004).

Identification of bacterial strains used in probiotic preparations continues to range from traditional microbiological plating and biochemical characterization methods to molecular typing techniques. The principal drawback of traditional microbiological techniques is their inability to differentiate between closely-related strains. Molecular typing techniques, such as 16S ribosomal analysis using DNA fingerprinting techniques or direct sequencing can be used to differentiate between closely-related species with high accuracy (Torriani et al. 2001; Heilig et al. 2002; Nielsen et al. 2003). The purpose of this research was to apply 16S sequencing techniques to evaluate seven probiotic products for bacterial content.

**Methods**

**Bacterial isolation and growth.** Bacterial isolates were obtained from seven probiotic products, including yogurts (Stonyfield Yogurt, Yoplait YoPlus, Dannon DanActive) and pills (GNC Super Acidophilus, Your Life Probiotic, Schiff Probiotic Acidophilus, and Probiohealth KE-99 Lacto). Ten milliliters of each yogurt was added to 90 ml of phosphate buffer (pH 7.2). The pills were dissolved in 100 ml phosphate buffer. Ten-fold dilutions were performed for each sample and dilutions ranging from $10^0$ to $10^6$ were plated on MRS agar with 0.05% L-Cys-HCl. The plates were incubated in an anaerobic chamber for 48 hours at 37°C. Single-colony isolates were used to inoculate 5 ml of MRS broth (with 0.05% L-Cys-HCl). Cultures were statically
incubated at 37°C for 48 hours and cells were harvested by centrifugation for ten minutes at 3000 rpm.

**DNA isolation.** Cell pellets were suspended in 200µl lysis buffer (50 mM Tris, pH 8, 50 mM EDTA, 1% SDS, 100 mM NaCl, 1% 2-mercaptoethanol), containing 0.5mg/ml Proteinase K and 20 mg/ml lysozyme. Cell suspensions were incubated at 45°C for one hour. Phenol–chloroform extraction was performed by adding phenol equal to the volume of cell suspension. The same amount of chloroform:isoamyl alcohol (24:1) was added and the tube was centrifuged at 14,000 rpm for one minute. The aqueous layer was transferred to a new tube and chloroform:isoamyl alcohol was added equivalent to two times the volume of aqueous solution. The solution was again centrifuged for one minute at 14,000 rpm. The aqueous layer was transferred to a new tube and ethanol precipitation was then performed by adding one-tenth the volume of 3M sodium acetate and 2.5 times the volume of 100% ethanol. The solution was incubated on ice for 20 minutes and the DNA pelleted by centrifugation at 14,000 rpm for ten minutes. The ethanol was pipetted off and 70% ethanol was added to rinse the pellet and the tube. The tube was centrifuged one minute at 14,000 rpm and the ethanol was pipetted off. The dried pellet was resuspended in TE buffer and the final concentration adjusted to 50 ng/µl.

**Genetic analysis.** PCR was performed on each sample to isolate and amplify the 16S region of the genome. The PCR reaction was carried out using 25 ng of the genomic template, 2x PCR Master Mix (Qiagen Inc., Valencia, CA), and universal primers designed from the conserved sequence of the 16S rRNA region (Table 1). The cycling conditions included an initial denaturation of 95°C for 5 minutes followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes.
Table 1. 16S PCR primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5’→ 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSF 8/20</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
</tr>
<tr>
<td>BSR 1541/20</td>
<td>AAGGAGGTGATCCAGCCGCA</td>
</tr>
</tbody>
</table>

Sequencing reactions were performed using 100 ng of 16S PCR DNA, Thermo Sequenase® sequencing kit (USB Corporation, Cleveland, OH), IRDye700 forward primer and IRDye800 reverse primer (Table 2). The primers were designed from conserved sequences of the 16S region and were located internal to the primers used in the previous PCR. The cycling conditions included an initial denaturation of 92°C for two minutes followed by 30 cycles of 92°C for 30 seconds, 54°C for 30 seconds, and 70°C for one minute.

Table 2. Sequencing primers for 16S locus

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’→ 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSF 349/17</td>
<td>IRDye700-AGGCAGCAGTGGGAAT</td>
</tr>
<tr>
<td>BSR 926/20</td>
<td>IRDye800-CCGTCATTTYTTTRAGTTT</td>
</tr>
</tbody>
</table>

Samples were then sequenced on a 5.5% acrylamide gel using a LI-COR 4300 Sequencer (LI-COR Inc, Lincoln, NE). The sequences were identified using the European Ribosomal Database BLAST search (Wuyts et al. 2004).

Results

Six of the seven probiotic formulations contained viable bacteria that grew under the conditions employed in this study. Within each plate the colonies appeared uniform, with the
exception of the GNC product which had two colonies that were visibly distinguishable by size and color. All of the colonies had morphology consistent with that of *Lactobacillus* bacteria. Colonies were round with distinct margins, mucoid and colored white to pale yellow. The bacteria exhibited a sweet smell that is characteristic of *Lactobacillus* bacteria.

Table 3. Results of 16S sequencing

<table>
<thead>
<tr>
<th>Product</th>
<th>Labeled Species Content</th>
<th>Species Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stonyfield Yogurt</td>
<td><em>L. acidophilus</em>, <em>B. bifidus</em>, <em>L. casei</em>, <em>L. reuteri</em>, <em>L. delbruekii subsp. bulgaricus</em>, <em>Streptococcus thermophilus</em></td>
<td><em>L. casei</em></td>
</tr>
<tr>
<td>Yoplait YoPlus</td>
<td><em>B. bifidum</em>, <em>L. delbruekii subsp. bulgaricus</em>, <em>S. thermophilus</em></td>
<td><em>L. delbruekii</em>, <em>S. thermophilus</em></td>
</tr>
<tr>
<td>Dannon DanActive</td>
<td><em>L. casei</em>, <em>L. delbruekii subsp. bulgaricus</em>, <em>S. thermophilus</em></td>
<td><em>L. casei</em></td>
</tr>
<tr>
<td>GNC Super Acidophilus</td>
<td><em>L. acidophilus</em>, <em>L. plantarum</em>, <em>B. bifidum</em>, <em>L. salivarius</em>, <em>L. rhamnosus</em>, <em>L. helveticus</em>, <em>S. thermophilus</em></td>
<td><em>L. acidophilus</em>, <em>L. plantarum</em>, <em>L. rhamnosus</em></td>
</tr>
<tr>
<td>Your Life Acidophilus Plus</td>
<td><em>L. acidophilus</em>, <em>B. bifidum</em>, <em>B. longum</em></td>
<td><em>L. sp. D79219</em></td>
</tr>
<tr>
<td>Schiff Probiotic Acidophilus</td>
<td><em>L. acidophilus</em></td>
<td><em>L. crispatus</em></td>
</tr>
<tr>
<td>Probiohealth KE-99 LACTO</td>
<td><em>L. casei</em></td>
<td>No viable bacteria</td>
</tr>
</tbody>
</table>

*Required yogurt starter cultures*
Stonyfield Yogurt claimed to contain six probiotic strains; however, *L. casei* was the lone species isolated. The yogurt starter cultures were not obtained even though both cultures were obtained from Yoplait YoPlus under identical growth conditions. Although both starter cultures were obtained from the Yoplait yogurt the primary probiotic strain, *B. bifidum* was not obtained. In contrast, the primary probiotic strain (*L. casei*) from the Dannon DanActive drink grew, but the two starter cultures did not (Table 3).

The highest number of bacterial strains obtained from a single product came from GNC Super Acidophilus. Three out of seven strains were identified (*L. acidophilus, L. plantarum, L. rhamnosus*). All of the colonies that grew from the Your Life pill were identical, and matched the database strain identified solely as *Lactobacillus species D79219*. Likewise, the Schiff pill had all identical colonies, which were identified as *L. crispatus*, even though the pill was labeled to contain *L. acidophilus*. The Probiohealth KE99 Lacto probiotic pill did not contain any viable bacteria despite numerous growth attempts under various growth conditions (Table 3). No *bifidobacteria* were able to grow under the conditions of this study.

**Discussion**

The growth conditions of this experiment were effective for obtaining viable *lactobacilli*, but were not ideal for *bifidobacteria* growth. MRS is not the recommended medium for *bifidobacteria*, although previous researchers have grown *bifidobacteria* on MRS (Nielsen et al. 2003; Temmerman et al. 2003).

The percentage of labeling accuracy in this study appeared lower than that reported in other papers; however it must be noted that the absence of certain bacteria strains in this study could be attributed not only to lack of viability of the products, but also to limitations of the
conditions of this experiment. There were, however, no particular deficiencies in this study’s methods that would explain the lack of growth.

The two standard yogurt fermentation starter cultures, *L. delbruekii subsp. bulgaricus* and *S. thermophilus*, were obtained from the Yoplait yogurt, but not from the other two yogurts, despite analysis under identical conditions. This suggests that, in this situation, the isolation of these bacteria was not hindered by the growth conditions but by the products themselves.

Identification of colonies based on their 16S ribosomal DNA sequence proved to be reliable and simple. The results are also reproducible. Despite the reliability of 16S sequencing, there is still difficulty distinguishing between very closely related strains of bacteria. 16S phylogeny studies demonstrate *L. acidophilus* and *L. crispatus* homology (Figure 1). This may explain why the isolates from the Schiff pill were identified as *L. crispatus* rather than *L. acidophilus* as listed on the label. Very few miscalled bases in a 16S sequence could result in incorrect identification. Manufacturers also likely encounter difficulty in differentiating between these very similar strains of *lactobacillus* and consequently sell the wrong bacteria. 16S homology may also explain the identification of the uncharacterized *L. sp. D79219* in the Your Life pill rather than *L. acidophilus.*
The KE99 Lacto pill did not contain any viable bacteria despite attempts to grow the bacteria at varying conditions. Some of the factors that affect viability of probiotic bacteria are shelf-life and storage conditions (Hamilton-Miller et al. 1999). This product was stored in a cool, dark space and was used within the shelf-life printed on the box, which suggests a deficiency of the product and not an error on behalf of the research methods.

Products that do not contain all or any of the bacteria on the label undermine the ability to demonstrate the efficacy of probiotic use. If consumers are misled by the labeling of probiotic products, it prevents them from making educated decisions about their consumption of live bacteria. It also raises concern for consumer safety if products contain bacteria that are not identified on the label. This study accentuates the need for manufacturers to reevaluate their methods for preparing and storing products with live bacteria and to reassess their quality control measures.
Literature Cited


