Detection and quantification of *Bifidobacterium lactis* LAFTI®B94 in human faecal samples from a consumption trial

Ping Su a,b,* And...
LAFTI®B94. These procedures were used to assess GI colonization and/or survival of B. lactis LAFTI®B94.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bifidobacterium lactis (B. lactis) strain LAFTI®B94 (provided by DSM Food Specialties, Australia) was used for method development and for detection of this strain in human faeces. Control strains used for method development were obtained from the culture collection of the Food Industry Innovation Collection, University of New South Wales, Australia. These included Bifidobacterium lactis Bb12 and Bifidobacterium longum B22. The Bifidobacterium strains were grown anaerobically at 37 °C in Reinforced Clostridial Medium (RCM, Oxoid, England) broth for 16 h or on RCM plates for 48 h.

2.2. Oligonucleotide primers and probes

The oligonucleotides used in this study are listed in Table 1. To design a probe for colony hybridization, all available bifidobacterial sequences of the 16S rDNA and the inter spacer regions were retrieved from the EMBL/GenBank database. Multiple alignments with the sequences were performed using the ANGIS software system operated by the Australian Genomic Information Centre, University of Sydney. Specific regions were identified for design of the probe Laf94p.

2.3. Subjects for the probiotic feeding trial and probiotic administration

The study population consisted of 5 healthy volunteers (4 female, 1 male), 25–50 years of age. No other probiotic products were consumed from 2 weeks before, during and 4 weeks after feeding with B94. B94 was consumed daily for one week, followed by a four-week washout period. The daily dose of B94 was $1 \times 10^{11}$ cells (freeze-dried powder in capsules; one capsule containing $5 \times 10^{10}$ cells, administered twice a day). The study was completed by all subjects. This study was approved by the UNSW HREC.

2.4. Preparation of faecal samples

Prior to the actual consumption trial, techniques were established for DNA isolation and detection from faecal samples. To test the sensitivity of the detection techniques, a dilution of the test bacteria ($10^2$–$10^9$ cells per g wet weight of faeces) was added to the faecal sample and quantified.

Faecal samples were taken before ingesting capsules on day 0, and after administration of B94 for 4 and 7 days. The dosage of probiotics was terminated on day 7. Faecal levels of B94 were also monitored for 28 days after termination of capsule ingestion. Altogether, it was analyzed twice during the feeding period and 6 times during the washout period. Faecal samples were placed on ice and analyzed within 0.1–4 h after defecation. Half-strength (10 ml) RCM broth was added per 0.5 g faecal sample and the sample was homogenized by vortexing at the highest speed. Aliquots (1.5 ml) were taken on ice and centrifuged at 500 rpm for 5 min at 4 °C and the supernatant was collected. The number of colony forming unit (CFU) was determined by serial dilution. Plates with a suitable number of colonies were used for colony hybridization. Residual supernatant was filtered through a pre-filter (Millex-AP20, Millipore) and the filtrate used for PCR analysis.

2.5. Colony hybridization

Colonies were transferred onto a nylon membrane by direct

Table 1
Sequences of PCR primers and probes used in colony hybridization

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence (5′–3′)</th>
<th>Length</th>
<th>Locationa</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>GAGGTTTGATCCTG</td>
<td>21</td>
<td>6–28 (16S rRNA)</td>
<td>[7]</td>
</tr>
<tr>
<td>Forward primer 338F</td>
<td>GCTCAG</td>
<td>18</td>
<td>321–338 (16S rRNA)</td>
<td>[8]</td>
</tr>
<tr>
<td>Reverse primer Lm3</td>
<td>AGT</td>
<td>21</td>
<td>1412–1432 (16S rRNA)</td>
<td>[9]</td>
</tr>
<tr>
<td>Reverse primer Bflact2</td>
<td>CGGTGCTNCCCACGT</td>
<td>18</td>
<td>991–1009 (16S rRNA)</td>
<td>[5]</td>
</tr>
<tr>
<td>Forward primer Bflact5</td>
<td>CCC</td>
<td>20</td>
<td>1651–1671 (16S–23S spacer region)</td>
<td>[5]</td>
</tr>
<tr>
<td>Reverse primer Laf94p</td>
<td>AATAC</td>
<td>26</td>
<td>16S–23S spacer region</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Corresponds to E. coli numbering of 16S rRNA gene [10].
colony lifts, followed by lysis, denaturation, neutralization and washing on a series of solution-saturated 3MM filter papers (Whatman). Nylon membranes were dried at 80 °C for 30 min, and the DNA was fixed to the membrane by UV cross-linking (5 min at 302 nm). Cell debris was removed by proteinase K treatment (80 U ml⁻¹, 37 °C, 1 h). The probe was labelled using a DIG oligonucleotide tailing kit. Prehybridization and hybridization were performed at 42 °C as described by the manufacturer. The DIG-labelled DNA–DNA hybrids were detected with a DIG luminescent detection kit. Images were developed on Lumi-Imager film (Roche).

2.6. PCR amplification

PCR for cultured cells was performed as described by Ventura et al. [5]. Each PCR reaction comprised 5 U of Taq Polymerase (Perkin–Elmer), 0.5 µl 10× PCR buffer, 2 µl of template (filtered sample), 25 mM dNTPs, and made up to 25 µl with Milli-Q water. The PCR conditions were modified for faecal samples from the trial as follows: cell lysis and DNA denaturation at 94 °C for 3 min, followed by 50 cycles of: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 7 min. The presence of B. lactis-specific PCR products was verified by 2% (w/v) agarose gel electrophoresis.

3. Results and discussion

3.1. Species-specific and genus-specific identification by PCR

Species-specific (for B. lactis, Bflact2–Bflact5), genus-specific (for bifidobacteria, P0-Lm3) and eubacteria-specific (P0-338F) conserved PCR primer pairs have been reported previously (Table 1). Therefore, the first step of the study was to test if the reported primer pairs could be used to detect L. lactis LAFTI® B94.

Genomic DNA was extracted from strains B. lactis LAFTI® B94, B. lactis Bb12 (positive control) and B. longum B22 (negative control) for PCR. A PCR product of 332 bp within the 16S rRNA gene of eubacteria was generated by P0-338F, indicating that all the samples were eubacterial in origin and that the quality of the DNA isolated was good enough for PCR analyses. A PCR product (680 bp) was generated by Bflact2–Bflact5 from B94 and Bb12, but not from B22 (data not shown). This indicated that the primers were species-specific and useful for identification of LAFTI® B94 in pure cultures.

The primer pairs were further tested for their applicability to detect B94 in spiked faecal samples. No PCR bands were detected in non-spiked faecal samples with the species-specific primer set, whereas the genus-specific primer pair yielded a discrete fragment of the expected size (1426 bp), indicating the presence of bifidobacteria in the faecal flora of the test subject (Fig. 1). Species-specific PCR bands could only be detected when cells of B94 or Bb12 were added to faeces before the isolation of total DNA (lanes 6 and 7, Fig. 1). This demonstrated that the species-specific primer pair could be used to detect B94 or Bb12 in sources as heterogeneous as faeces.

To determine the detection limit of PCR for these experiments, various quantities of B94 cells were mixed with the faecal sample. For species-specific primers, B94 could only be detected when it was added at a concentration of at least 10⁴ cells per g (wet weight) of faeces. The presence of colony lifts, followed by lysis, denaturation, neutralization and washing on a series of solution-saturated 3MM filter papers (Whatman). Nylon membranes were dried at 80 °C for 30 min, and the DNA was fixed to the membrane by UV cross-linking (5 min at 302 nm). Cell debris was removed by proteinase K treatment (80 U ml⁻¹, 37 °C, 1 h). The probe was labelled using a DIG oligonucleotide tailing kit. Prehybridization and hybridization were performed at 42 °C as described by the manufacturer. The DIG-labelled DNA–DNA hybrids were detected with a DIG luminescent detection kit. Images were developed on Lumi-Imager film (Roche).

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To determine the detection limit of PCR for these experiments, various quantities of B94 cells were mixed with the faecal sample. For species-specific primers, B94 could only be detected when it was added at a concentration of at least 10⁴ cells per g (wet weight) of faeces. It was also found that the filtration treatment of faecal samples with a pre-filter increased the purity of the PCR template, and was able to increase the sensitivity of the test.

3.2. Development of colony hybridization for quantitative detection of B94

A rapid quantification method is necessary to detect viable B. lactis LAFTI® B94 in human faecal samples. A colony hybridization technique was developed for this purpose. The DIG oligonucleotide tailing system, using terminal transferase to add a long fluorescent tail to the 3′-end of the probe, allowed the use of a small probe and improved hybridization signals.

Initially, the primer Bflact5 was tested as a probe in colony hybridization, but it failed to hybridize. A new probe, Laf94p (26 bp), was designed from a comparative analysis of all bifidobacterial 16S–23S spacer regions. Additional mismatches were introduced into the probe to give a 23% difference compared to the closest species, thus giving better specificity.

After colony hybridization and colorimetric detection with the DIG-luminescent detection kit, typical images

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**Fig. 1.** Establishment of the PCR method using faecal samples spiked with different test bacteria. Lanes 1, 6 and 11, Bb12 added; lanes 2, 7 and 12, B. lactis LAFTI® B94 added; lanes 3 and 8 and 13, B22 added; lane 4, 5, 9, 10, 14 and 15, faeces only. Lanes 1–5, PCR reactions using an eubacteria-specific primer pair (P0-338F); lanes 6–10, PCR reactions using a species-specific primer pair (Bflact2–Bflact5); lanes 11–15, PCR reactions using a genus-specific primer pair (P0-Lm3); lane 16, 100 bp DNA ladder.
were obtained (Fig. 2). B94 colonies, which resulted from spiked faecal samples, generated distinct signals, whereas colonies from human faecal samples without spiked B94 samples were completely clear (Fig. 2). This indicated that colony hybridization with probe Laf94p is suitable for detection of B94 in human faecal samples.

3.3. Specific detection of B94 in human faeces from a consumption trial

A consumption trial was carried out to assess the sensitivity and specificity of the probe for detection of B94. Faecal samples were collected and prepared as described. Both PCR amplification and colony hybridization were performed on the samples. Signals were not detected in samples before the feeding period, indicating that B94 was absent (or below the detection level) in the volunteers’ faeces prior to feeding. On the third feeding day, B94 was found in high numbers in all samples (Fig. 3). The numbers of B94 colonies were highest on the seventh day of feeding (up to $1.8 \times 10^9$ cfu g$^{-1}$ wet weight, Fig. 3). The B94 population declined after feeding ceased (washout period, days 8–35, Fig. 3). One week after termination of feeding, B94 was still detected in 4 of the 5 subjects (Fig. 3). Two weeks after termination of feeding, B94 was detected in 2/5 subjects (Fig. 4). Four weeks after termination of feeding, B94 could still be detected in one subject (Fig. 3).

In a recently published paper, it was reported that B. lactis Bb12 could not be detected one week after feeding stopped [6]. Our results suggested that either B94 may colonize longer in the GI, or that the method of detection described here is more sensitive, or that the dosage used in the two trials was different. Our results indicate a longer-term colonization with B94 in one subject (>4 weeks). B94 utilizes a range of prebiotics, including soybean-oligosaccharides and resistant starch [4]. Studies are currently underway to investigate the effects of prebiotic carbohydrates on the survival and colonization of B94 in the GI tract.

![Fig. 2. Colony hybridization of B94 using Laf94p, from pre-consumption trial samples. (a) faecal sample spiked with B94; (b) faecal sample only.](image)

![Fig. 3. GI survival of B94 in consumption trial faecal samples. Viable counts of B94 were determined by colony hybridization with probe Laf94p. PCR reactions were carried out when viable counts dropped below $10^2$ cfu g$^{-1}$ wet faeces.](image)

![Fig. 4. PCR analysis on consumption trial faecal samples collected two weeks after termination of feeding. Lane 1, 100 bp DNA ladder; lane 2, B94 colony as control; lanes 3–7, subjects 1–5.](image)
References


