INTRODUCTION

As population demographics shift towards a more elderly society (WHO, 2002), the incidence of functional gastrointestinal disorders is becoming more prevalent (Greenwald, 2004; Hébuterne, 2003; Pilotto, 2004). This, coupled with an increasing dependence on conventional antibiotics and the emergence of antibiotic-resistant bacteria, poses significant challenges to the effective treatment of gastrointestinal disorders. However, the potential prophylactic and therapeutic properties attributed to certain probiotic bacteria may offer an effective alternative (Sleator & Hill, 2007a). Indeed, numerous clinical studies have attributed a myriad of impressive health-promoting effects to probiotics, including effective treatment of certain digestive and metabolic disorders (Leahy et al., 2005) as well as antagonistic activities against microbial pathogens (Asahara et al., 2001, 2004; Gagnon et al., 2006; Servin, 2004; Silva et al., 1999).

This study focuses on the probiotic strain Bifidobacterium breve UCC2003, the genome of which has recently been sequenced (S. C. Leahy and others, unpublished data). While studies concerning the clinical efficacy of strain UCC2003 are at a preliminary stage, recent studies have identified a number of potential health-promoting properties (Coakley et al., 2003), making it a potentially important probiotic culture. However, while genomic analysis has identified a number of stress adaptation systems employed by B. breve UCC2003 (Ventura et al., 2004a, 2005a, b, c, d, e, 2006), the genome appears devoid of obvious compatible solute-uptake systems. Found in numerous prokaryotic and eukaryotic species, compatible solute accumulation has been shown to protect a variety of cell types under a range of stressful conditions by maintaining cytoplasmic protein and membrane integrity (Abee & Wouters, 1999; Sleator et al., 2000, 2001b, 2003a, b; Sleator & Hill, 2002).

In order to survive and proliferate within the gastrointestinal tract, probiotics must tolerate several environmental hurdles, including the low pH of the stomach, as well as reduced water activity (a_w) and bile in the upper small intestine. Furthermore, the ability to persist in the intestine is considered to be a valuable criterion in achieving optimal probiotic efficacy (Vaughan et al., 2002). While many bifidobacteria have evolved to tolerate the stresses of gastrointestinal transit (Asahara et al., 2004; Fujiwara et al., 2001; Gagnon et al., 2006; Picard et al., 2005), significant variation exists among different strains. Thus, the application of processes to improve the physiological robustness of probiotic cultures in vivo is a clinical imperative (Sleator & Hill, 2006, 2007b, c).
Pathogenic species, with life cycles bridging the host and external milieu, have evolved strategies to ensure their continued survival in these diverse ecological niches (Hill et al., 2002; Sleator & Hill, 2002, 2005). Thus, pathogenic genera represent a useful reservoir of stress survival mechanisms which could potentially improve the physiological robustness and clinical effectiveness of probiotic strains. The term 'patho-biotechnology', coined by Sleator & Hill (2006), describes the exploitation of pathogenic stress-survival strategies in the design of more versatile probiotic cultures (Sleator & Hill, 2006, 2007b, c).

Previously the in vitro stress tolerance of the probiotic strain Lactobacillus salivarius UCC118 was significantly improved by heterologous expression of the betaine uptake system BetL, isolated from Bifidobacterium breve (Sheehan et al., 2006). Herein, we describe a similar approach to improve the gastric transit, gastrointestinal persistence and systemic BetL, isolated from B. breve UCC2003 was performed as described by MacConaill et al. (2003). Essentially, mid-exponential-phase cells were chilled on ice for 20 min and this was followed by centrifugation. The cell pellet was washed twice and resuspended in 0.5 M sucrose/1 mM citrate buffer (pH 5.8). The cells were incubated on ice for 10 min followed by electrottransformation with a Bio-Rad Gene Pulser II apparatus under the following conditions: 25 μF, 200 Ω and 2.0 kV cm⁻¹. RCM was added to the cells, and the mixture was incubated anaerobically at 37 °C for 2.5 h prior to plating.

Plasmid construction. PCR primers with incorporated PstI (5' - CATCTGAGGCTTCTCCGCATTTCGCTC-3') and XbaI (5' - CAACTAGACCTATCAATTACGCCATTTCC-3') restriction enzyme sites (underlined) were used to amplify the complete betL gene from the chromosome of L. monocytogenes LO28. The resultant PCR product was digested with PstI and XbaI and subsequently ligated into similarly digested pNZ8048 using T4 DNA ligase (Roche Diagnostics). The resultant plasmid, containing betL under the transcriptional control of its own promoter, was designated pNZ8048betL. Both pNZ8048betL and pNZ8048 (control) were initially introduced into E. coli MKH13, and plasmid DNA extracted from successful transformants was sequenced and subsequently transformed into B. breve UCC2003, giving rise to UCC2003-BetL⁺. B. breve UCC2003 harbouring pNZ8048, used as a negative control, was designated UCC2003n.

Transcriptional analysis. B. breve UCC2003 cells were grown at 37 °C to mid-exponential phase in RCM. Cells were harvested by centrifugation and flash-frozen at −80 °C with liquid nitrogen. Total RNA was extracted using the hot acid phenol procedure described by Ripio et al. (1998), and cDNA was synthesized by adding 1 μg total RNA to 4 μl 5 × RT buffer (Roche), 2 μl 100 mM dithiothreitol, 0.5 μl of a deoxynucleoside triphosphate mix, 0.25 μl RNasin, 100 ng of the random primer p(dN)₆, and 1 μl Expand reverse transcriptase (Roche). The reaction mixture was incubated at 42 °C for 9 h.

Table 1. Bacterial strains and the plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties*</th>
<th>Source or reference</th>
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<tr>
<td>Strains</td>
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<td>B. breve</td>
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<td>UCC2003</td>
<td>Wild-type parent strain</td>
<td>UCC culture collection</td>
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<tr>
<td>UCC2003n</td>
<td>UCC2003 containing the cloning vector pNZ8048</td>
<td>This study</td>
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<tr>
<td>UCC2003-BetL⁺</td>
<td>UCC2003 containing the cloning vector pNZ8048betL</td>
<td>This study</td>
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<td>E. coli</td>
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<td>MKH13</td>
<td>MC4100Δ(putPA)101Δ(proP)2Δ(proU)</td>
<td>Kempf &amp; Bremer (1995)</td>
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<td>L. monocytogenes</td>
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<td>LO28</td>
<td>Serotype 1/2c</td>
<td>P. Cossart, Institut Pasteur, Paris</td>
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<td>EGD-e</td>
<td>Sequenced strain</td>
<td>UCC culture collection</td>
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<td>Plasmids</td>
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<td>pNZ8048</td>
<td>Cm⁺</td>
<td>De Ruyter et al. (1996)</td>
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<tr>
<td>pNZ8048betL</td>
<td>Cm⁺, harbouring betL under the control of the listerial promoter</td>
<td>This study</td>
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*Cm⁺, chloramphenicol resistant.
In all cases, control PCR reactions were used to ensure the complete removal of DNA from RNA preparations prior to reverse transcription. Oligonucleotide primers betLF (5’-AGAGGAAAGGATGATCCCTGCCCTTTAGTGC-3’) and betLR (5’-CACACATTGGATTTCCCGTAGTGTT-3’) were used for PCR amplification of a 314 bp betL DNA fragment from UCC2003-BetL⁺. All glass and plastic-ware used in RNA analysis was first treated with 2 % SDS for 15 min, before rinsing with 1:10 in diethyl pyrocarbonate (DEPC)-treated water.

**Resistance to gastric acid and low pH.** Simulated gastric juice was prepared by suspending pepsin (0.3 % w/v) (Sigma) in saline (0.5 %, w/v). The solution, with or without 5 mM added betaine (Sigma), was adjusted to pH 2.5 with HCl and filter-sterilized through a 0.45 μm membrane (Charteris et al., 1998). Also, RCM was adjusted to pH 2.5 using HCL. Overnight cultures of B. breve UCC2003-BetL⁺ and UCC2003n were inoculated (3 %) into the simulated gastric juice and pH-modified RCM. Both solutions were incubated anaerobically at 37 °C. Viable cell counts were performed by diluting cultures in quarter-strength Ringer’s solution and enumerating on reinforced clostridial agar (RCA).

**Osmotolerance.** Overnight cultures of B. breve UCC2003-BetL⁺ and UCC2003n were washed twice in Ringer’s solution before being inoculated (2 %) into DM and DM supplemented with 1.5 % (w/v) NaCl or 6 % (w/v) sucrose, both of which represent an osmolarity equivalent to that encountered in the upper small intestine (Gupta & Chowdhury, 1997). Strains were grown statically at 37 °C and OD₆₀₀ readings taken over 35 h. Optical density values were confirmed by viable plate counts.

**Bile tolerance.** Human bile, obtained from the gallbladder of patients undergoing laparoscopic cholecystectomy, was filter-sterilized through a 0.45 μm membrane before being added to cooled sterilized RCM. The concentration (1:10 dilution), pH 5.5 and osmolarity (0.3 M NaCl) of human bile were adjusted to mimic the environment of the upper small intestine, into which bile is secreted. Overnight cultures of B. breve UCC2003-BetL⁺ and UCC2003n were inoculated (3 %) into the bile preparation. Samples were incubated anaerobically at 37 °C and viability monitored by diluting cultures in one-quarter-strength Ringer’s solution and enumerating onto RCA.

**Survival of B. breve derivatives following exposure to successive stress treatments mimicking in vivo conditions.** Overnight cultures of B. breve UCC2003-BetL⁺ and UCC2003n were centrifuged, washed with PBS and resuspended in simulated gastric juice pH 2.5 for 90 min. Cells were then harvested by centrifugation and resuspended in human bile, adjusted to the osmolarity of the gut, for 90 min. Experiments were performed in triplicate with and without 5 mM added betaine. Viable cell counts were performed by diluting cultures in quarter-strength Ringer’s solution and enumerating onto RCA.

**Survival of B. breve UCC2003 derivatives in the murine model.** Overnight cultures of B. breve strains UCC2003-BetL⁺ and UCC2003n were centrifuged, washed with PBS and used to inoculate 8-12-week-old female BALB/c mice, five per treatment group. Colonization of bifidobacteria was established by three consecutive daily administrations whereby each animal received 20 μl of ~5 × 10⁹ cells using a micropipette tip placed immediately behind the incisors (Sleator et al., 2001a). In vivo survival of B. breve UCC2003 derivatives was monitored by periodic examinations of viable counts in faecal pellets. Briefly, fresh faecal pellets extracted by scuffing each animal were weighed, homogenized in quarter-strength Ringer’s solution, diluted and plated onto RCA/Cm plates. Thirty-two days post-infection mice were euthanased and the bifidobacterial numbers in the intestines and caecum of infected animals were determined by spread-plating homogenates onto RCA/Cm plates. PCR primers specific to the B. breve 16S rRNA region [biFF (5’-AGAGGAAAGGATGATCCCTGCCCTTTAGTGC-3’) and biFR (5’-TTTTTGTGAGGTTCGGATTCTGCTGTCAGGATGA-3’); S. C. Leahy and others, unpublished data] were used to confirm that Cm⁻ bifidobacteria isolated from the faecal pellets were indeed B. breve, while PCR primers specific to betL (betLF and betLR) confirmed the presence of the gene in UCC2003-BetL⁺. Furthermore, faecal pellets taken before administration with bifidobacteria showed no background microflora when plated on the antibiotic selection plates.

**Virulence assays.** Colonization of B. breve UCC2003 derivatives was established by three successive daily oral administrations as described above. Mice in the control group received PBS. On the fourth day, L. monocytogenes EGD-e was pelleted by centrifugation, washed with PBS, and used to orally inoculate the animals with ~10⁵ c.f.u. ml⁻¹. On the same day, faecal samples were taken from each animal and the numbers of recoverable UCC2003-BetL⁺ and UCC2003n were assessed. PCR was used to confirm that Cm⁻ bifidobacteria isolated from the faecal pellets were B. breve UCC2003. Three days post-infection animals were sacrificed, individual livers and spleens were homogenized in PBS, and serial dilutions were plated onto BHI/Cm agar plates.

**Statistical analysis.** Treatment comparisons were performed using ANOVA test at 95 % significance, using the Systat software version 9 (SPSS).

### RESULTS

**betL expression analysis**

RT-PCR analysis gave a single 314 bp band for UCC2003-BetL⁺, indicating active transcription of the betL gene in the complemented strain (Fig. 1). As expected, no band was observed for the control strain UCC2003n lacking betL.

![Fig. 1. RT-PCR analysis of betL expression in UCC2003-BetL⁺.](image)

(a) Control PCRs using cDNA template and 16S rRNA-specific primers biFF and biFR confirm that equal levels of RNA were extracted for UCC2003n and UCC2003-BetL⁺. (b) No products were obtained when RNA was used as template with biFF and biFR, confirming no DNA carryover. (c) betL-specific primers betLF and betLR amplify a 314 bp fragment from UCC2003-BetL⁺ cDNA, while as expected no product was obtained when the cDNA of the control strain UCC2003n was used as template. All bands are the result of 22 PCR cycles.
Control PCRs on total cDNA from both UCC2003-BetL⁺ and UCC2003n using the 16S rRNA bifF and bifR primers gave bands of equal intensity, confirming that equal levels of RNA were extracted for the control and complemented strains. However, when total RNA was used as a template no product was obtained, confirming the absence of any chromosomal DNA.

**Tolerance of *B. breve* UCC2003 to gastric acid is significantly improved by the addition of betL**

Survival of *B. breve* UCC2003-BetL⁺ and UCC2003n was monitored following exposure to simulated gastric juice adjusted to pH 2.5 in the presence and absence of 5 mM added betaine (Fig. 2). UCC2003-BetL⁺ was recovered at significantly (*P*<0.05) higher levels than UCC2003n, 2 and 3 h post-exposure in the presence of betaine. No significant differences were detected in the absence of betaine. Neither strain (UCC2003-BetL⁺ or UCC2003n) was detected following 30 min exposure to RCM adjusted to pH 2.5 (data not shown).

**BetL enhances the osmotolerance of *B. breve* UCC2003**

Betaine accumulation has long been associated with an increase in osmotolerance. Initially, growth of UCC2003-BetL⁺ and UCC2003n in DM at 37 °C was compared to assess whether the presence of betL had an effect on the growth rate in the absence of additional NaCl (Fig. 3a). No difference in the growth rate or the extent of growth was observed, suggesting that the presence of betL has no impact under normal, non-stress growth conditions. However, with 6% added sucrose (Fig. 3b), UCC2003-BetL⁺ displayed a faster growth rate and reached a higher final optical density than UCC2003n. Similarly, when both strains were exposed to DM supplemented with 1.5% NaCl (Fig. 3c), UCC2003-BetL⁺ again displayed a significantly faster growth rate than UCC2003n and also reached a high final optical density.

**The presence of betL in *B. breve* UCC2003 does not provide protection against bile**

When cultures were challenged with human bile, betL was unable to enhance the tolerance of *B. breve* UCC2003 to the biological detergent beyond that achieved by the control strain, UCC2003n. Both UCC2003-BetL⁺ and UCC2003n displayed similar levels of resistance to human bile, showing a 1.5 log reduction in c.f.u. after 6 h exposure (data not shown).
**B. breve UCC2003-BetL** is recovered at higher levels than UCC2003n following successive stress treatments mimicking in vivo conditions

UCC2003-BetL was recovered at higher levels than UCC2003n after exposure to a simulated gastric juice followed immediately by exposure to human bile adjusted to mimic conditions encountered in vivo (Fig. 4). The only significant differences were observed following exposure to the successive stress treatments in the presence of 5 mM added betaine, UCC2003-BetL remaining viable at significantly \((P<0.05)\) higher levels than the control strain UCC2003n. The slightly higher survival levels observed for UCC2003-BetL in the absence of added betaine most likely result from betaine pre-accumulation during inoculum preparation.

**Recovery of B. breve UCC2003 derivatives from faecal samples, intestines and caecum of BALB/c mice**

Following oral inoculation of BALB/c mice with **B. breve** UCC2003-BetL or UCC2003n for three consecutive days, both strains could be recovered from the murine intestine. A population greater than \(10^6\) c.f.u. g\(^{-1}\) was recorded in the faecal contents for up to 32 days (Fig. 5a). Viable counts of UCC2003-BetL were significantly \((P<0.05)\) greater than those of UCC2003n during the initial 3 days of monitoring post-feeding. UCC2003-BetL and UCC2003n were recovered at similarly high levels between days 6 and 10, after which time a significant \((P<0.001)\) decrease in UCC2003n numbers was noted between days 10 and 14. Colonization by **B. breve** UCC2003n stabilized on day 14, with no significant decline in cell numbers recorded from then until the completion of the trial. However, while counts of UCC2003-BetL also stabilized at day 14, this strain was recovered in numbers significantly \((P<0.05)\) greater than UCC2003n from day 14 onwards. On day 32, mice were sacrificed and the levels of both strains in the small and large intestines and caecum were assessed (Fig. 5b). UCC2003-BetL was recovered at significantly \((P<0.01)\) higher levels in the large intestine and caecum when compared to UCC2003n.

**Listerial infection in the organs of mice is reduced following consumption of B. breve UCC2003 harbouring betL**

Consistent with previous observations, higher levels of UCC2003-BetL were recovered from the faecal pellets following oral inoculation, suggesting that BetL improves the gastrointestinal persistence of the probiotic (data not shown). In order to ascertain whether the higher colonization rates would enhance or compromise the therapeutic benefits of the strain, the extent of listerial infection in the livers (Fig. 6a) and spleens (Fig. 6b) of mice fed UCC2003-BetL, UCC2003n and PBS were compared. While the numbers of organ-specific c.f.u. recovered from the livers did not differ significantly, listerial proliferation in the spleen was significantly \((P<0.05)\) lower in mice fed **B. breve** UCC2003-BetL in comparison to the two control groups. It is therefore reasonable to conclude that **B. breve** UCC2003-BetL demonstrates enhanced clinical efficacy when challenged with *L. monocytogenes*.

**DISCUSSION**

In order for bifidobacteria to exert a probiotic effect, it is essential that they remain viable within the delivery vehicle (food or tablet formulation), survive passage through the gastrointestinal tract and reach the intestine in sufficiently high numbers (Dunne et al., 2001; Tuomola et al., 2001). Previously we described the successful expression of betL in *Lb. salivarius* UCC118 (Sheehan et al., 2006) and in *E. coli* (Sleator et al., 1999, 2003c), demonstrating significantly improved survival of both strains to stresses encountered in foods and food-processing environments. In the present study we report the potential of the approach to enhance the gastrointestinal persistence and clinical efficacy of **B. breve**, a clinically important probiotic culture.

Survival of **B. breve** was significantly enhanced by the presence of betL when exposed to a simulated gastric juice pH 2.5. This is the first evidence that betL may play a role in improving probiotic viability during transit through the stomach. Interestingly, when cultures were exposed to a
complex medium (RCM) adjusted to a similar pH (using HCl), no significant difference in the acid tolerance of the strains was observed, implying that pH alone is not responsible for the improved viability of UCC2003-BetL+ in simulated gastric juice. Furthermore, UCC2003 derivatives declined at a significantly faster rate in the complex broth compared to the simulated gastric juice. Previously, Saarela et al. (2005) observed that pepsin protects bifidobacteria at pH 2.5, suggesting that the reduced toxicity associated with the simulated gastric juice, as opposed to RCM, might be attributed to the presence of pepsin. In any case, improved survival of the complemented strain in gastric juice containing added betaine suggests that it is the compatible solute (accumulated via BetL) that is responsible for the observed increased tolerance to the gastric juice. Interestingly, in support of this observation Termont et al. (2006) recently reported improved tolerance to gastric juice in a Lactococcus lactis strain expressing trehalose-synthesizing genes from E. coli, suggesting a protective role for compatible solutes in the gastric environment.

Once in the upper small intestine, probiotics are exposed to low a_w (equivalent to 0.3 M NaCl) (Gupta & Chowdhury, 1997) and bile salts (at a concentration of 5 mM) (De Boever & Verstraete, 1999). Consistent with our previous observations with Lb. salivarius UCC118 (Sheehan et al., 2006), a significant osmoprotective effect was observed following the introduction of betL into B. breve, facilitating growth of the probiotic in conditions similar to those encountered in vivo (1.5% NaCl and 6% sucrose, both of which approximate the osmolarity of the gut). The presence of betL increased the growth rate of the probiotic and enabled the transformed strain to reach a higher final optical density compared to the control. As well as elevated osmolarity, the biological detergent bile also poses a significant hurdle to microbes in the upper small intestine (Sleator et al., 2005; Sleator & Hill, 2007d). However, betL appeared to offer no protection to B. breve challenged with human bile, a result not altogether surprising given that BetL, or indeed betaine, has not previously been linked to bile tolerance. Finally, combining the gut-associated stresses in a sequence mimicking that encountered in vivo...

**Fig. 5.** Recovery of B. breve UCC2003-BetL+ (○) and UCC2003n (●) from female BALB/c mice over 32 days of analysis. (a) Faeces for bacteriological analysis were obtained from five mice in each treatment group and viable counts of B. breve UCC2003 derivatives were determined as described in the text. (b) Recovery of B. breve UCC2003-BetL+ (white bars) and UCC2003n (black bars) from the small and large intestine and the caecum of the murine model after 32 days. The error bars represent the standard error of five animals (n=5). Asterisks represent significant differences.

**Fig. 6.** Listerial infection in the livers (a) and spleens (b) of BALB/c mice. Animals were fed ~10^9 c.f.u. ml^-1 of either UCC2003-BetL+ or UCC2003n for three consecutive days. The control group was fed PBS. On the fourth day, all animals were infected with ~10^11 c.f.u. ml^-1 L. monocytogenes EGD-e. Three days post-listerial infection the animals were sacrificed and the numbers of listeria were determined. Asterisks represent significant differences.
confirmed that UCC2003-BetL+ survived significantly better than the control strain in conditions similar to those encountered during gastric transit.

Following in vitro studies, in vivo analysis using BALB/c mice demonstrated stable intestinal colonization of B. breve UCC3003 derivatives following oral inoculation. A possible explanation for the initial differences observed in the faecal pellets (days 1–3) may be greater numbers of UCC3003-BetL+ isolates surviving gastric transit, a consequence of the greater resistance to gastric fluid. Once clear of the stomach, the elevated osmolarity of the intestinal tract likely promotes increased growth and survival of the BetL-complemented strain relative to the control. These findings correlate with previous observations by Sleator et al. (2001a) in which another compatible solute-uptake system (OpuC; facilitating both carnitine and betaine uptake) in L. monocytogenes was identified as an essential element for optimal intestinal survival of Listeria. Significantly higher levels of UCC2003-BetL+ were recovered from the colon, the recognized environmental niche for bifidobacteria. However, no significant differences in recoverable B. breve UCC2003 derivatives were observed in the small intestine, a finding that most likely reflects the fact that the small intestine is rich in bile, against which we have shown BetL offers no protective effect over and above that of the wild-type.

It seems reasonable to correlate increased delivery of probiotics to the intestine with improved therapeutic function, but this remained to be confirmed in vivo. A number of studies have reported that intestinal infections in the murine model can be prevented or alleviated by precolonization of the intestine by B. breve strain Yakult. Furthermore, feeding B. breve strain Yakult to mice with a disrupted microbiota resulted in a large number of pro-inflammatory cytokines and increased growth and survival of the BetL-complemented strain. This was confirmed that UCC2003n showed no obvious decline in listerial numbers relative to those administered PBS, B. breve UCC2003 complemented with betL had a significant effect on listerial infection, particularly in the spleen. This is significant, given that splenic infection is an indicator of total systemic infection. Furthermore, damage to the spleen further weakens the body’s immune response, increasing the mortality rate in severe infections. To our knowledge, this is the first clear evidence of an enhanced therapeutic effect following precise bio-engineering of a probiotic strain.

While we recognize that the introduction of genes from pathogens into probiotic cultures is unlikely to meet with immediate approval from regulatory authorities, this study proves that the concept of using such an approach to design more versatile cultures is at least scientifically valid. Following proof of concept, it is entirely likely that BetL homologues from GRAS (generally regarded as safe) organisms might offer similar protective effects. Furthermore, natural selection of probiotic cultures with elevated expression of such homologues might ultimately dispense with the necessity for recombinant DNA approaches altogether.

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