Improving gastric transit, gastrointestinal persistence and therapeutic efficacy of the probiotic strain *Bifidobacterium breve* UCC2003

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Given the increasing commercial and clinical relevance of probiotic cultures, improving their stress tolerance profile and ability to overcome the physiological defences of the host is an important biological goal. In order to reach the gastrointestinal tract in sufficient numbers to exert a therapeutic effect, probiotic bacteria must resist the deleterious actions of low pH, elevated osmolarity and bile salts. Cloning the listerial betaine uptake system, BetL, into the probiotic strain *Bifidobacterium breve* UCC2003 significantly improved probiotic tolerance to gastric juice and conditions of elevated osmolarity mimicking the gut environment. Furthermore, whilst stable colonization of the murine intestine was achieved by oral administration of *B. breve* UCC2003, strains harbouring BetL were recovered at significantly higher levels in the faeces, intestines and caecum of inoculated animals. Finally, in addition to improved gastric transit and intestinal persistence, this approach improved the clinical efficacy of the probiotic culture: mice fed *B. breve* UCC2003-BetL⁺ exhibited significantly lower levels of systemic infection compared to the control strain following oral inoculation with *Listeria monocytogenes*.

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).

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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 *Listeria monocytogenes* (Sheehan *et al.*, 2006). Herein, we describe a similar approach to improve the gastric transit, gastrointestinal persistence and therapeutic efficacy of *Bifidobacterium breve* UCC2003. This study thus provides the first *in vivo* experimental evidence in support of the patho-biotechnology concept.

METHODS

Bacterial strains, media and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Bifidobacterium breve* UCC2003 (previously designated NCIM B8807), originally isolated from an infant nursing stool (Ventura *et al.*, 2004b), was routinely cultured in reinforced clostridial medium (RCM) at 37 °C in an anaerobic chamber (Don Whitley Scientific). Where mentioned, a defined medium (DM) based on that described by Kongo *et al.* (2003) was used to culture *B. breve*, with glycine betaine (Sigma) being added as a filter-sterilized solution. *Escherichia coli* MKH13 was grown in Luria-Bertani (LB) medium at 37 °C with agitation. *L. monocytogenes* was grown with aeration at 37 °C in brain heart infusion (BHI) broth. A concentrated stock of the antibiotic chloramphenicol (Cm) was prepared (Maniatis *et al.*, 1982) and added to media at a concentration of 4 µg ml⁻¹ and 7.5 µg ml⁻¹ when selecting for bifidobacteria and *Listeria* respectively. All media were supplied by Oxoid.

Genetic manipulations. Plasmid DNA isolation was performed using the Qiagen QIAprep spin miniprep kit. PCR was performed using a Hybaid PCR Express system. Oligonucleotide primers for PCR were synthesized by Sigma-Genosys Biotechnologies, and *Taq* DNA polymerase (Biotaq; Bioline) was used for all reactions. Colony PCR was carried out following lysis of cells at 96 °C for 10 min with IGEPAL CA-630 (Sigma). *E. coli* was transformed by standard methods (Maniatis *et al.*, 1982) while electrotransformation of *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 electrotransformation 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 *Pst*I (5'-CATCTGCAGGCTTCTCCCCCTTTTCCTC-3') and *Xba*I (5'-CATTCTAGAGCTCTATTCCAATTACCGCCATTTC-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 *Pst*I and *Xba*I 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 pNZ8048*betL*. Both pNZ8048*betL* 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

Strain or plasmid	Relevant properties*	Source or reference
Strains		
<i>B. breve</i>		
UCC2003	Wild-type parent strain	UCC culture collection
UCC2003n	UCC2003 containing the cloning vector pNZ8048	This study
UCC2003-BetL ⁺	UCC2003 containing the cloning vector pNZ8048 <i>betL</i>	This study
<i>E. coli</i>		
MKH13	MC4100Δ(<i>putPA</i>)101Δ(<i>proP</i>)2Δ(<i>proU</i>)	Kempf & Bremer (1995)
<i>L. monocytogenes</i>		
LO28	Serotype 1/2c	P. Cossart, Institut Pasteur, Paris
EGD-e	Sequenced strain	UCC culture collection
Plasmids		
pNZ8048	Cm ^r	De Ruyter <i>et al.</i> (1996)
pNZ8048 <i>betL</i>	Cm ^r , harbouring <i>betL</i> under the control of the listerial promoter	This study

*Cm^r, 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'-AATCGTGCTTCCCGC-TTTAGTC-3') and *betLR* (5'-GCACACTTGCTATTCCCGCTTG-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 on 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'-AGAAAGGAGGT-GATCCAGCCGCAC-3') and bifR (5'-TTTTGTGGAGGGTTTCG-ATTCTGGCTCAGGATGA-3'); S. C. Leahy and others, unpublished data] were used to confirm that Cm^r 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 ~10¹¹ 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^r 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⁺. (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 biffR 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. 2. Survival of *B. breve* UCC2003-BetL⁺ (circles) and UCC2003n (triangles) in simulated gastric juice pH 2.5, in the presence (open symbols) and absence (closed symbols) of 5 mM added betaine. Standard error of triplicate experiments was determined for each data point.

Fig. 3. Growth of *B. breve* UCC2003-BetL⁺ (circles) and UCC2003n (triangles) in DM (a) at 37 °C under normal physiological conditions, with 6% (w/v) added sucrose (b) and with 1.5% (w/v) added NaCl (c) in the presence (open symbols) and absence (filled symbols) of 5 mM added betaine. Both 1.5% NaCl and 6% sucrose represent an osmolarity equivalent to that encountered in the upper small intestine. Cell growth was measured spectrophotometrically by determining OD₆₀₀. Each point represents the mean $\pm$ SEM of at least three independent experiments.

(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

Fig. 4. Survival of *B. breve* UCC2003-BetL⁺ (white bars) and UCC2003n (black bars) (a) with and (b) without 5 mM added betaine in simulated gastric juice for 90 min, mimicking conditions encountered in the stomach, followed by exposure to human bile for 90 min, representing conditions encountered in the upper small intestine. Asterisks represent significant differences.

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.

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. 6. Listerial infection in the livers (a) and spleens (b) of BALB/c mice. Animals were fed $\sim 10^9$ 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 $\sim 10^{11}$ 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 osmolality 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 the administration of bifidobacteria that alter the microecology of the gut (Asahara *et al.*, 2001, 2004; Gagnon *et al.*, 2006). Asahara *et al.* (2001) demonstrated that antibiotic-induced proliferation and translocation of *Salmonella enterica* serovar Typhimurium into the host is markedly inhibited 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 probiotics colonizing the intestine for a period of at least 2 weeks (Asahara *et al.*, 2004). Moreover, infection with Shiga-toxin-producing *E. coli* O157:H7 was dramatically inhibited. Both studies suggested that the pH-lowering effects of the probiotic appeared to be important for enabling these anti-infectious activities. Furthermore, *B. breve* strains have been reported to display antimicrobial activity against pathogens through competitive exclusion (Lievin *et al.*, 2000), whereby probiotic strains with similar adherence mechanisms inhibit the attachment and colonization of the pathogen at these binding sites, thus protecting the host from infection (Marco *et al.*, 2006).

In the present study, we report for the first time that feeding *B. breve* UCC2003-BetL⁺ prior to listerial administration can protect the animal from infection. While mice administered 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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