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ORIGINAL ARTICLE

The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics

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Abstract

Aims: To conduct *in vitro* and *in vivo* assessments of the safety of two species of *Bacillus*, one of which, *Bacillus subtilis*, is in current use as a food supplement.

Methods and Results: Cultured cell lines, Caco-2, HEp-2 and the mucus-producing HT29-16E cell line, were used to evaluate adhesion, invasion and cytotoxicity. The Natto strain of *B. subtilis* was shown to be able to invade and lyse cells. Neither species was able to adhere significantly to any cell line. The Natto strain was also shown to form biofilms. No strain produced any of the known *Bacillus* enterotoxins. Disc-diffusion assays using a panel of antibiotics listed by the European Food Safety Authority (EFSA) showed that only *Bacillus indicus* carried resistance to clindamycin at a level above the minimum inhibitory concentration breakpoints set by the EFSA. *In vivo* assessments of acute and chronic dosing in guinea pigs and rabbits were made. No toxicity was observed in animals under these conditions.

Conclusions: *Bacillus indicus* and *B. subtilis* should be considered safe for oral use although the resistance of *B. indicus* to clindamycin requires further study.

Significance and Impact of the Study: The results support the use of *B. subtilis* and *B. indicus* strains as food supplements.

Introduction

The increasing interest in the use of bacteria as probiotics has prompted a number of organizations to recommend guidelines for their use (FAO/WHO 2002; Sanders 2003). The rationale for closer scrutiny has been brought on by a number of factors: first, reports of infection that have been correlated with probiotic consumption, which includes members of the lactic acid bacteria (LAB) (FAO/WHO 2002; Borrielo *et al.* 2003) and *Bacillus*, although all have occurred in patients with underlying medical conditions; second, the production of enterotoxins in some products, notably *Bacillus cereus*; and finally, the mislabelling and incomplete characterization of bacterial species.

Less well known than the lactobacilli and bifidobacteria are certain species of the spore-forming *Bacillus* genus that are being used as probiotics, notably *Bacillus clausii*, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus coagulans* (often mislabelled as '*Lactobacillus sporogenes*') and *B. cereus* (Sanders *et al.* 2003; Hong *et al.* 2005). The use of *B. subtilis* is approved for use as a food supplement in at least one European country (Italy), but for other species, this is not the case with the exception of *B. clausii* that is licensed as a prophylactic medicine in the product 'Enterogermina' (manufactured by Sanofi-Aventis, Milan, Italy). The use of *Bacillus* species as probiotics raises the question of safety as a number of species are known to be pathogenic, most notably the members of the *B. cereus* group that includes *Bacillus anthracis* and *B. cereus*, *Bacillus thuringiensis*, *Bacillus pseudomycoides* and *Bacillus weihenstephanesis. Bacillus cereus* is a well-documented food-poisoning micro-organism where illness results from the production of one or more of a number of enterotoxins (Granum and Lund 1997; Granum 2002; Guinebretiere *et al.* 2002; From *et al.* 2005). Pathogenicity however

is strain specific as some varieties of *B. cereus* exist that produce no enterotoxins and indeed, as mentioned earlier, some are in current use as probiotics for both human and animal use. The illnesses caused by strains of *B. thuringiensis* and *B. weihenstephanesis* are less well understood but probably are similar to those produced by *B. cereus* and are caused by the production of enterotoxins.

Regarding the other *Bacillus* species, far less is known, although a number of reports have associated *Bacillus* spp. to clinical conditions (de Boer and Diderichsen 1991; Osipova *et al.* 1998; Salminen *et al.* 1998; Sanders *et al.* 2003; Logan 2004). The incidence of these *Bacillus*-related illnesses is low, and in many cases, they can be accounted for by misdiagnosis where 'spores' are recovered from clinical samples. Opportunistic infections have also been reported, for example, in immunocompromised patients, but such infections can often be found with members of other 'nonpathogenic' genera. However, a number of reports have shown that isolates of some *Bacillus* species exist that appear to carry toxigenic characteristics, for example, one or more genes that could encode an enterotoxin have been found in species outside of the '*B. cereus* group' (Rowan *et al.* 2001; Phelps and McKillip 2002; From *et al.* 2005). Finally, it appears that the dose of bacteria consumed can also play an important factor in developing the illness (Kramer *et al.* 1982; Duc *et al.* 2005). In the case of *Bacillus* species, there is clearly a need for strains to be evaluated on a case-by-case basis.

In this work, we have examined the safety of *Bacillus indicus* strain HU36 compared with two strains of *B. subtilis*, PY79 and Natto. PY79 is a laboratory strain derived from the 168 type strain (Youngman *et al.* 1984). Natto is a variety of *B. subtilis* that is used in the fermentation of the Japanese fermented soybean staple known as 'Natto'. The Natto strain has a long history of use as part of a food product and has been shown to contain more than 10^8 viable bacteria per gram (Hosoi and Kiuchi 2004). HU36 is a pigmented isolate of *B. indicus* and is a member of a subgroup of *Bacillus* spp. that are rich in carotenoids (Duc *et al.* 2006). At least 11 different carotenoids are found in the walls of HU36 spores and the vegetative bacterium, and consumption of HU36 spores could provide a useful source of dietary carotenoids. Our extensive study has included *in vivo* experiments demonstrating that there appears to be no obvious risk associated with the consumption of these organisms.

Materials and methods

Bacterial strains and cell lines

HU36 is a yellow-orange pigmented spore-forming strain of *B. indicus*, which has been described previously (Duc *et al.* 2006). PY79 is a prototrophic strain of *B. subtilis* derived from the 168 type strain (Youngman *et al.* 1984). Natto is *B. subtilis* var. *Natto* and was obtained from a sample of the Japanese staple, 'Natto' (Hosoi and Kiuchi 2004). SC2329 is a toxin-producing strain of *B. cereus* (Hoa *et al.* 2000), and for invasion studies, a laboratory strain of *Listeria monocytogenes* (NCTC 19115) was used as an invasive control. Caco-2 and HEp-2 cell lines were obtained from the European Collection of Cell Lines. The mucus-secreting cell line, HT29-16E, was provided by Dr R. La Ragione (Veterinary Laboratories Agency, Weybridge, UK).

General methods and preparation of spores

Purified suspensions of spores were made by the exhaustion method (Nicholson and Setlow 1990) using Difco sporulation medium (DSM). Spore suspensions were lysozyme treated and then heat treated (68°C 1 h) to remove residual vegetative cells and stored as aliquots at −20°C prior to use.

Analysis of enterotoxins and virulence traits

Methods to detect putative *B. cereus* enterotoxin genes from *Bacillus* species by PCR amplification from chromosomal DNA have been reported elsewhere (Duc *et al.* 2004, 2005). Primer sets were those described by Guinebretiere *et al.* (2002). The Hbl and Nhe enterotoxins were detected and measured using commercial kits; the BCET-RPLA (*Bacillus cereus* enterotoxin reversed passive latex agglutination) kit (Oxoid) to detect Hbl and the Tecra BDE kit (Tecra Diagnostics, Sydney, Australia) to detect the Nhe enterotoxin. Haemolysis was detected by streaking on sheep blood agar plates and lecithinase by streaking colonies on *B. cereus* selective agar containing egg yolk and 48 h incubation at 37°C.

Surfactin

The surfactin produced by the bacteria is measured by the method described by Youssef *et al.* (2004).

Adhesion and invasion studies

Strains were grown in brain heart infusion (BHI) broth (Oxoid) at 30°C for 15–18 h and washed two times with phosphate-buffered saline (PBS) (note that bacteria grown in BHI do not form spores). Bacteria were then suspended to *c.* 10^7–10^8 CFU ml^{-1} in complete cell culture medium supplemented with HEPES (using optical density readings to approximate CFU). Adherence and invasion to HT29-16E [a mucus-secreting cell line; (Augeron and

Laboisse 1984; Lesuffleur *et al.* 1990)], Caco-2 and HEp-2 cells were determined using the methods described by Rowan *et al.* (2001) but with minor modifications. In brief, cells were seeded in 24-well culture plates (10^5 cells per well). Cell monolayers were grown for a minimum of 2 days (Caco-2 and HEp-2) or 7 days (HT29-16E) at 37°C, 5% CO_2, using complete minimal essential medium (MEM) containing 10% foetal calf serum for Caco-2 and HEp-2 cells, and complete Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal calf serum for HT29-16E cells.

Prior to assays, the cell monolayers were washed once with complete culture medium, and then inoculated with 1 ml of 10^7–10^8 CFU bacteria per well (providing a bacteria to cell ratio of *c.* 100 : 1) in complete culture medium supplemented with HEPES (0·01 mol l^{-1}) in triplicate, and incubated for 2 h at 37°C in a 5% CO_2. Control wells (without cell lines) were added with the same number of bacteria and incubated in parallel. After incubation, the cell monolayers were gently washed four times with culture medium to remove any nonadherent bacteria. The total number of adherent and invasive bacteria in each well was counted by lysing cells with 1 ml of 0·1% Triton X-100 in water for 5 min at 37°C. Next, the viable count was made on DSM agar plates (or BHI agar for NCTC 19115). The percentage of adherent and invasive bacteria was calculated by comparing CFU with control wells.

Invasion assays were done parallel to the adherence assay. After the washing steps, 1 ml of complete culture medium containing 100 μg ml^{-1} of gentamicin was added to each well and incubation continued for a further 2 h at 37°C. Live bacteria are sensitive to gentamicin and so any bacteria that adhered to the cells and present in the medium would be killed, thus allowing a measurement of only bacteria that had invaded the cells. Cell monolayers were then washed three times with culture medium to remove gentamicin, and the number of invaded bacteria in each well counted by lysing cells with 0·1% triton in water. Measurements of adhesion are those that had values for invasion subtracted.

Cytotoxicity assay

The assay was done as described by Rowan *et al.* (2001) using HEp-2, Caco-2 or HT29-16E cell monolayers seeded at 5×10^4 cells per well. Cells were infected with filter-sterilized (0·2 μm) supernatants from overnight cultures (BHI, 30°C) of bacteria to be tested. Samples of supernatant (0·1 ml) were added to cultured cells (in triplicate) immediately after either trypsin treatment (0·1% trypsin; 5 min) or heat treatment (95°C, 10 min). Monolayers containing the bacterial supernatants were incubated overnight at 37°C in a 5% CO_2 atmosphere. After overnight incubation, the suspension from each well was removed and 90 μl of fresh complete medium + 10 μl WST-1 cell proliferation reagent (Roche Diagnostics) added. Samples were incubated for 4 h at 37°C in 5% CO_2. Optical densities of the suspensions at 450 nm were then measured using a Rosys anthos HT3 ELISA reader and cytotoxicity (% of dead cells) calculated as (1 – optical density of test sample/optical density of negative control) × 100.

Anaerobic growth

The procedure to evaluate the ability of *Bacillus* strains to grow and sporulate under anaerobic conditions was as described elsewhere (Tam *et al.* 2006).

Persistence studies

A single oral dose (0·2 ml) of 1×10^9 spores was given to mice (pathogen-free, Balb/c, females, age 6 weeks) by intragastric gavage. Heat-resistant CFU per gram obtained from freshly collected faeces was determined as described by Duc *et al.* (2004). Note that mice carry a low level of aerobic and heat-resistant spore formers in their faeces at a level below 10^3 CFU g^{-1}. Careful observation of heat-resistant survivors from mouse faeces enables the counting of 'administered' spores but only at levels above 10^3 CFU g^{-1} of faeces where they can be identified by their colony appearance. These experiments were performed under the UK Home Office project licence PPL 70/6126.

Simulated intestinal conditions

Spores and vegetative cells were evaluated for resistance to simulated gastric fluids (SGF) using the method described in Barbosa *et al.* (2005).

Biofilm formation

Biofilm formation was determined using the method of Fall *et al.* (2004) using growth (37°C for 2–3 days) using CMK liquid medium, CMK agar (2%), CM agar (1%), CMK agarose (0·3%) or CM agarose (0·3%).

Antibiotic testing

Antibiograms for strains were obtained by the disc diffusion method according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS 1997). The minimum inhibitory concentration (MIC) values were obtained by the serial antibiotic dilution in MH (Mueller Hinton) broth.

Production of antimicrobials

Two methods were used.

1. The spotting method. *Bacillus* strains were inoculated as a spot (*c.* 5–10 mm) on the surface of an MH agar plate. After 72 h at 37°C, the bacteria were killed by exposure to chloroform vapour for 15 min, and the plates were overlaid with MH agar containing 24- to 48-h-old indicator strains at 10^8 CFU ml^{-1}. The plates were incubated for 24 h at 37°C. The antagonistic activity was detected as the presence of a growth inhibition zone around the spot.

2. The agar well diffusion method. Six millimetre diameter wells were cut into MH agar plates containing a lawn of the indicator strain and then filled with 100 μl of cell-free-culture supernatants from the *Bacillus* strains grown in MH medium. The plates were incubated at 37°C for 24–48 h and zones of inhibition measured.

Indicator strains included three Gram-positive and two Gram-negative strains and were *Staphylococcus aureus* CIP 20256, *Listeria innocua* CIP 8011, *B. subtilis* 168, *Salmonella indiana* CA11 and *Escherichia coli* CIP 54·8.

In vivo toxicity studies

All the *in vivo* experiments were carried out at the, GLP (Good Laboratory Practice)-compliant, Quality Control department of the National Institute of Vaccines and Biological Substances (IVAC) in Nha Trang, Vietnam. Approval was provided by the Vietnam Ministry of Health and were performed in compliance with the United States Food and Drug Administration guidelines on animal testing. Animal numbers were designed to use the smallest numbers of animals possible but sufficient to yield meaningful information and are based on a number of recent papers that have evaluated the *in vivo* safety of probiotic bacteria (Zhou *et al.* 2000a; Sorokulova *et al.* 2007).

Short-term continuous exposure study in rabbits

Eighteen New Zealand White rabbits (male, 3 months old) divided into three groups of six animals with each animal housed individually. Two groups were orally dosed (by gavage) with 1 ml of a purified suspension of spores (1×10^9 spores per ml^{-1}). One group received HU36 spores and the other Natto. The naïve group received 1 ml of saline. Animals were dosed using this regime daily for 30 days. On day 30, blood was withdrawn (by cardiac puncture from anaesthetized animals) for haematological analysis and animals were then killed humanely, and samples of different visceral organs and tissues were collected for histological analysis, including liver, kidneys, spleens, small intestines and mesenteric lymph nodes.

Acute single-dose study on guinea pigs

Thirty guinea pigs (Harley Dunkin, male and females, 5 weeks old) were used. A single 1 ml dose of 1×10^{12} spores of either HU36 or Natto was administered orally by gavage to groups of five animals. Each animal was housed individually. A naïve group received 1 ml of saline. Animals were observed daily for behaviour, appearance, activity and faeces for 14 days. Body weights were recorded on days 0, 7, 14 and 17. On day 17, blood was withdrawn (by cardiac puncture from anaesthetized animals) for haematological analysis and animals were then killed humanely. Samples of different visceral organs and tissues were collected for histological analysis, including liver, kidneys, spleens, small intestines and mesenteric lymph nodes.

Histology

Samples of organs and tissues were fixed in 10% formalin, transferred into ethanol solutions with increasing concentrations and embedded in paraffin wax. Tissue sections were cut at 6 μm and stained with haematoxylin and eosin (H&E).

Haematology

Blood samples were obtained from rabbits and guinea pigs by cardiac puncture and total red blood cells (RBC), leucocytes, haemoglobin concentration and differential percentages of white blood cells were determined.

Statistics

The data were analysed using the Mann–Whitney *U*-test. For all tests, the level of significance was set at $P < 0{\cdot}05$. Unless indicated otherwise, values in the text are means ± SEM.

Plasmid analysis

Plasmid DNA was extracted from the *Bacillus* strains using the method of Voskuil and Chambliss (1993).

Results

Growth and sporulation properties

The ability of HU36, Natto and PY79 to grow and sporulate was examined (data not shown). HU36 was unable to grow under anaerobic conditions in contrast to PY79 and Natto that could. Natto was able to sporulate under anaerobic conditions. In the presence of nitrate in the medium as much as 6% of the CFU could form spores in contrast to PY79, which produced very low levels of spores ($<8 \times 10^{-3}$) under anaerobic conditions. The

ability of *Bacillus* species to form biofilms was assessed by a variety of methods, including liquid growth and on solid media (data not shown). Neither HU36 nor PY79 could form biofilms by any of the methods tested. Natto however could form hydrophobic biofilms in CMK liquid and on 2% CMK agar but not on 1% CM agar. *Bacillus cereus* SC2329 was able to form biofilms on 1% CM agar but not in other growth media. Both Natto and SC2329 produced dendritic growth on CM agarose (0·3%) and profuse surface growth on CMK agarose (0·3%). The resistance of spores to simulated intestinal fluids (SIF) was also measured. Incubation of spores of PY79, Natto or HU36 with SGF showed no significant effect (i.e. less than 1 log difference) in viability after 1 h incubation at 37°C. Incubation of spores with SIF for 3 h at 37°C also showed no significant drop in spore viability (data not shown). Vegetative cells of the three strains were also examined in SFG and SIF and were all extremely sensitive to these fluids with none showing greater than 0·001% after 60 min incubation (data not shown).

Antibiotic resistance

HU36 was evaluated for its resistance to a panel of antibiotics, including those highlighted by the European Food Safety Authority (EFSA 2005) and recommended by the NCCLS (1997). Antimicrobial resistance was determined in two ways: first, using the agar disc-diffusion assay (Table 1); and second, by establishing the MIC (Table 2). PY79 exhibited MIC at breakpoint levels for streptomycin and tetracycline and Natto for streptomycin. HU36 was found to carry noticeable resistance only to clindamycin, which was above the published MIC breakpoint for this compound (EFSA 2005). To determine whether this resistance is acquired, we attempted to isolate plasmid DNA from HU36 and PY79, but failed to isolate any DNA that might correspond to episomal DNA (data not shown). We also examined the ability of HU36 to inhibit the growth of selected Gram-positive and Gram-negative bacteria (*S. aureus*, *B. subtilis*, *L. innocua*, *S. indiana* and *E. coli*) using spot and agar well-diffusion tests. Using appropriate positive controls, HU36 and PY79 were found to have no inhibitory activity, although Natto showed significant inhibition of *L. innocua*, *B. subtilis* 168 and *E. coli*.

Persistence of spores in the mouse gastrointestinal tract (GIT)

The shedding of spores of HU36, PY79 and the *B. cereus* strain SC2329, in the faeces of mice that were given a single oral dose of 1×10^9 spores was measured (Fig. 1). Counts of PY79 and HU36 spores were no longer within detectable levels after 12 days, while SC2329 persisted for 3 weeks.

Table 1 Antibiotic resistance profiles of *Bacillus* strains

Antibiotic discs*	PY79†	Natto†	HU36†
Ampicillin (10)*	23·6 ± 0·9† S	38·2 ± 1·5 S	20·2 ± 3·5 S
Streptomycin (10)	15·5 ± 1 I	17 ± 2 S	25·3 ± 3 S
Erythromycin (15)	25·6 ± 1·5 S	32·2 ± 1·8 S	30·6 ± 1·2 S
Tetracycline (30)	19·2 ± 1·5 S	32 ± 2 S	35 ± 5 S
Chloramphenicol (30)	25 ± 1·4 S	25 ± 2 S	28·6 ± 2·5 S
Rifampicin (30)	20 ± 1 S	37 ± 2 S	34 ± 3 S
Ciprofloxacin (5)	30·6 ± 0·4 S	36 ± 1·5 S	36 ± 3 S
Clindamycin (2)	18·6 ± 0·5 S	23·1 ± 1 S	6·3 R
Vancomycin (30)	23·6 ± 2 S	26 ± 1 S	31 ± 1 S
Trimethoprim (5)	26 ± 1·3 S	32·5 ± 2 S	30 ± 2 S
Gentamicin (10)	26·3 ± 1 S	30 ± 1 S	36 ± 1 S
Neomycin (30)	21·6 ± 0·5 S	24·5 ± 1·5 S	29 ± 2·5 S
Trimethoprim + sulfamethoxazol‡	27 ± 1 S	33 ± 1·5 S	35 ± 2 S
Enrofloxacin (5)	28·3 ± 3 S	35 ± 1 S	36·6 ± 1·5 S
Kanamycin (30)	25·5 ± 1·5 S	30 ± 3 S	36·3 ± 2 S
Linezolid (30)	28·5 ± 3 S	36 ± 1·5 S	37·6 ± 0·5 S

*Antibiotic-impregnated discs (6 mm) with amount, in μg shown in brackets.
†Diameter of inhibition from three individual experiments. S, sensitive; I, intermediate;
R, resistant.
‡Trimethoprim (1·25)/sulfamethoxazole (23·75).

Adhesion and invasion assays

Adhesion of HU36, PY79, Natto and SC2329 to Caco-2 and HEp-2 cells in addition to a mucus-secreting cell line, HT29-16E, was evaluated using *in vitro* methods (Fig. 2). HT29-16E cells exhibit differentiation features characteristic of mature intestinal cells, including the secretion of mucin, and are therefore presumably more informative than Caco-2 cells (Augeron and Laboisse 1984; Lesuffleur *et al.* 1990; Devine *et al.* 1992). In each

Table 2 Minimum inhibitory concentration (MIC)

Antibiotic	MIC (mg l^{-1}) PY79	Natto	HU36	*Bacillus* EFSA Breakpoints*
Ampicillin	<0·1	<0·1	<0·1	NR
Vancomycin	<0·1	0·5	< 0·1	4
Gentamicin	0·5	<0·1	<0·1	4
Kanamycin	0·5	1	<0·1	8
Streptomycin	8	8	0·5	8
Neomycin	0·5	1	<0·1	8
Erythromycin	<0·1	<0·1	<0·1	4
Clindamycin	1	1	16	4
Quinuprintin-Dalfopristin	2	2	0·5	4
Tetracycline	8	0·5	<0·1	8
Chloramphenicol	2	4	2	8
Trimethoprim	1	1	1	8
Linezolid	NT	NT	NT	4
Rifampicin	0·5	0·5	<0·1	4

EFSA, European Food Safety Authority; NR, breakpoint not required; NT, not tested.
*Strains with MIC higher are considered resistant. Breakpoints are outlined in EFSA reports (EFSA 2005).

case, a suspension of *c.* 10^7–10^8 vegetative cells of each strain was incubated for 2 h with the cultured cell line, after which the number of adhering cells was measured. In terms of the levels of binding, our results were in general agreement with a previous study (Rowan *et al.* 2001) that characterized the binding of *Bacillus* strains to cultured cell lines. HU36 was shown to be the least adherent strain in all cell lines. With HEp-2 cells, both Natto and PY79 bound at reasonably high levels (1–1·5%) and significantly ($P < 0·05$) greater than the

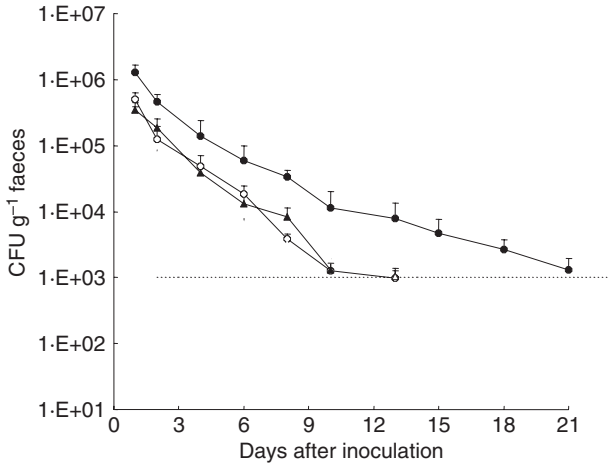

Figure 1 Persistence of HU36 in the mouse GIT. Counts of spores excreted in the faeces of mice following a single, fixed oral dose (1×10^9) of spores HU36 (▲), PY79 (○) and SC2329 (●). Heat-resistant counts were determined from fresh faeces collected from individual mice as described previously (Duc *et al.* 2004).

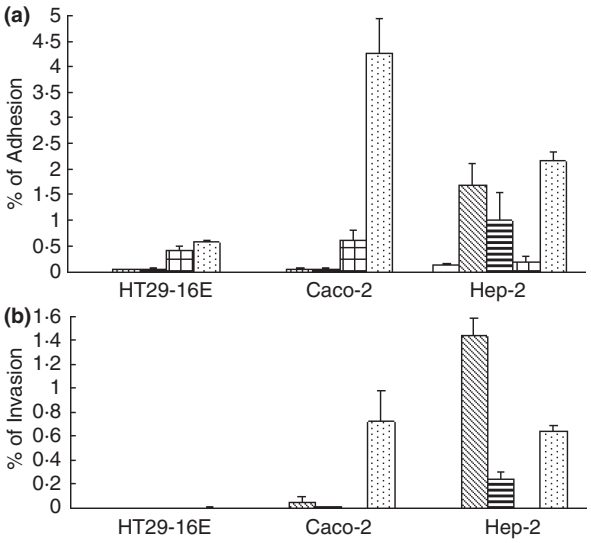

Figure 2 Adhesion and invasion HT29-16E, Caco-2 and HEp-2 cells. Vegetative cells of HU36 (*Bacillus indicus*), Natto (*Bacillus subtilis*), PY79 (*B. subtilis*), SC2329 (*Bacillus cereus*) were added to the cell lines at a concentration of 10^7–10^8 CFU per well and an approximate ratio of bacteria to cells of 100. After 2 h incubation at 37°C, the total number of adherent (a) and invasive (b) bacteria were determined as described in materials and methods and expressed as a percentage. Values shown are representative of triplicate samples from separate trials and shown as means with standard errors. (□), HU36 (B.i); ▨, Natto (B.s); ▤, PY79 (B.s); ▥, SC2329 (B.c); ▩, NCTC 19115 (L.m).

B. cereus strain SC2329. Invasion was also measured using a strain of *L. monocytogenes* (NCTC 19115) as a positive control. HU36 and SC2329 failed to invade any cell line but PY79 and Natto did show some invasion to both Caco-2 and Hep-2 cells but not HT29-16E. In HEp-2 cells, Natto showed levels of invasion significantly ($P < 0·05$) higher (∼1·44%) than the *L. monocytogenes* control (0·65%).

Potential virulence factors

PCR was used to evaluate the presence of known *B. cereus* enterotoxin genes in the chromosome of HU36, PY79, Natto, and as a control, a toxin-producing strain of *B. cereus*, SC2329 (Table 3). This method has been used previously to profile putative food-poisoning *Bacillus* strains (Guinebretiere *et al.* 2002; Phelps and McKillip 2002; Duc *et al.* 2004, 2005). Our results showed that no strain, other than SC2329, carried a known *B. cereus* enterotoxin gene. *In vivo* analysis was also made for the Hbl and Nhe *B. cereus* enterotoxins, and all strains tested negative. Haemolysis was not produced on sheep's blood agar by HU36, PY79 and Natto. Production of lecithinase (a phospholipase) was also negative in HU36 and PY79, but a weak reaction was observed with Natto. Finally,

Table 3 Potential virulence characteristics

Characteristic	*Bacillus* strains			
	HU36	PY79	Natto	SC2329
Haemolysis*	γ	γ	γ	β
Lecithinase†	–	–	±	+
Surfactin‡	–	–	++	–
Hbl complex§				
hblA	–	–	–	+
hblB	–	–	–	–
hblC	–	–	–	+
hblD	–	–	–	+
HBL enterotoxin¶	0	0	0	128
Nhe complex§				
nheA	–	–	–	+
nheB	–	–	–	+
nheC	–	–	–	+
NHE enterotoxin**	1	1	1	4
Other enterotoxin genes§				
cytK	–	–	–	+
bceT	–	–	–	+

*Haemolysis on sheep blood agar: β, complete haemolysis with a clear zone around colonies; γ, no changes.

†Lecithinase production: +, blue precipitation of hydrolysed lecithin around peacock blue colonies (indicative of *Bacillus cereus*); –, no changes; ±, weak colouration.

‡Using the oil-spreading technique (Youssef *et al.* 2004): - is no measurable activity and ++ represents a diameter of oil displacement of 16–20 mm.

§Gene encoding components of the Hbl or Nhe enterotoxins or other *B. cereus* enterotoxins were diagnosed by PCR: +, a PCR product of the expected size was amplified; –, no PCR product was detected.

¶Production of the Hbl enterotoxin in growing cells was determined using the BCET-RPLA toxin kit (Oxoid) and expressed as an index where a value of 0 is negative according to the manufacturer's instructions. The sensitivity of the test is 2 ng ml^{-1}.

**Production of the Nhe enterotoxin in growing cells was measured using the 'Bacillus Diarrhoeal Enterotoxin Visual Immunoassay kit' (Tecra Diagnostics). According to the manufacturer's instructions, strains with an index of <3 are considered negative and the sensitivity of the test is 1 ng ml^{-1}.

surfactin activity was measured using a simple oil-displacement method (Table 3). Only Natto was shown to produce surfactin using this method in agreement with previous studies of this lipopeptide from the Natto strain (Nagal *et al.* 1996).

Cytotoxicity assays

The toxicity of supernatants of growing cultures of HU36, PY79, Natto and SC2329 to cultured cell monolayers was evaluated using a spectrophotometric assay to measure cell death. Our results shown in Table 4 demonstrated that HU36 supernatants contained a very low level of activity that was both heat and trypsin stable. PY79 showed no toxicity to Caco-2 or HEp-2 cells but some activity with HT29-16E cells. By contrast, Natto and SC2329 carried a significant level of a heat-sensitive toxic material in their cell-free supernatants.

In vivo toxicity assessments

In a short-term continuous-exposure study, rabbits received daily oral doses of 10^9 spores of HU36 or Natto for 30 days. With this continuous dosing regime, there were no adverse effects, neither on the general health status of the animals nor their feed intake (data not shown). Translocation of pathogenic bacteria from the GIT to the MLN (mesenteric lymph nodes) and then dissemination to other organs can result in bacteremia. For this reason, histology was used to examine selected visceral organs and tissues, and no changes in selected visceral organs and tissues were observed (data not shown). No significant differences in the haematological indexes were observed in blood from control and treated rabbits (Table 5).

For an acute toxicity study, guinea pigs were chosen because they are considered the most sensitive laboratory animals. A single oral dose (1×10^{12} CFU spores) of HU36 or Natto spores was administered to each animal. Appetite, behaviour, faeces and weight gain are the most general sensitive indicators of health status, and there were no noticeable abnormalities 17 days after the administration of spores in their feed intake (data not shown). Weight gains are shown in Fig. 3 and no significant

Table 4 Effect of heat or trypsin treatment on cytotoxicity of cell-free supernatants on HEp-2, Caco-2 and HT29-16E epithelial cells

Bacillus spp.	% of cells dead after the indicated treatment*								
	HEp-2			Caco-2			HT29-16E		
	Normal	HT	TT	Normal	HT	TT	Normal	HT	TT
PY79	0	0	0	0	0	0	26 ± 1	10 ± 5	17 ± 7
Natto	67 ± 4	1 ± 9	60 ± 4	53 ± 4	0	49 ± 2	54 ± 5	11 ± 4	49 ± 3
SC2329	68 ± 10	16 ± 9	62 ± 11	69 ± 4	0	64 ± 1	54 ± 3	24 ± 6	56 ± 5
HU36	9 ± 4	18 ± 10	6 ± 9	0	0	0	2 ± 6	1 ± 5	1 ± 3

*Supernatant fluids were untreated (normal), heat treated (HT) or trypsin treated (TT).

Table 5 Haematology measurements (mean ± SEM)

Parameter	Subchronic toxicity (rabbits)*			Acute toxicity (guinea pigs)†		
	Control‡	Natto	HU36	Control‡	Natto	HU36
Red blood cells × 10^{12}	5·26 ± 0·37	4·73 ± 0·69	5·8 ± 0·21	4·76 ± 0·397	4·86 ± 0·37	4·78 ± 0·35
Haemoglobin (g l^{-1})	118 ± 14·20	131·05 ± 13·7	109·44 ± 8·50	128·42 ± 7·50	131·92 ± 4·79	132·90 ± 5·52
Haematocrite (l l^{-1})	0·343 ± 0·03	0·39 ± 0·02	0·37 ± 0·02	0·369 ± 0·027	0·38 ± 0·024	0·39 ± 0·018
Platelets × 10^{9}	383 ± 110·7	370·35 ± 93·52	594·81 ± 30·23	391·56 ± 66·02	379·80 ± 91·9	334·94 ± 56·25
White blood cells × 10^{9}	4·49 ± 1·61	4·38 ± 1·73	5·95 ± 0·92	3·59 ± 0·57	4·09 ± 0·50	3·30 ± 0·28
Neutrophils (%)	63·8 ± 3·25	56·11 ± 3·00	60·21 ± 3·27	53·00 ± 5·60	52·86 ± 5·80	54·05 ± 2·45
Lymphocytes (%)	32·15 ± 1·63	36·6 ± 1·80	34·31 ± 2·26	40·88 ± 2·77	39·77 ± 4·71	36·63 ± 5·69
Monocytes (%)	2·05 ± 1·72	3·65 ± 1·52	2·84 ± 0·86	5·28 ± 4·35	6·36 ± 4·53	5·02 ± 3·42
Eosinophils (%)	0·87 ± 0·06	1·54 ± 1·13	1·76 ± 0·05	1·36 ± 0·94	0·82 ± 0·59	3·60 ± 0·63
Basophils (%)	1·23 ± 0·42	1·85 ± 0·32	2·40 ± 0·02	0·3 ± 0·08	0·54 ± 0·16	1·22 ± 0·28

*Day 30 samples in rabbits dosed orally, every day for with for 30 days with 1 × 10^{9} spores.
†Day 17 samples in guinea pigs administered with a single oral dose of 1 × 10^{12} spores.
‡A naïve group receiving no spores.

differences ($P > 0·05$) were seen between male or female animals receiving Natto and HU36 spores. Comparison of treated animals and those of the control group did reveal some differences at a significant level ($P < 0·05$). These were at day 7 in the female groups receiving HU36 spores at days 7, 14 and 17, and in the female group at day 14 for those receiving Natto spores. Histological analysis of organs and tissues revealed no signs of inflammation or pathological changes (data not shown) and no differences in the haematological indexes measured in blood from control and treated rabbits (Table 5).

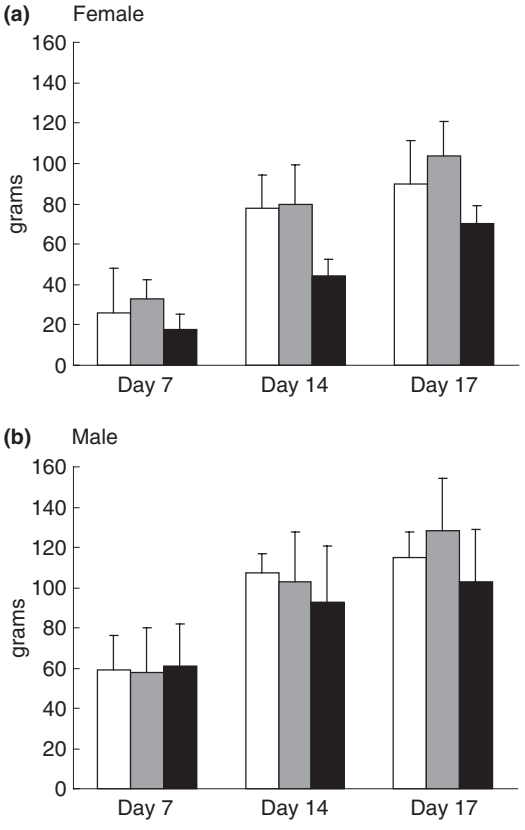

Figure 3 Guinea pig growth rates. Average weight gains (g) of groups of guinea pigs receiving a single oral dose of 1 × 10^{12} spores are shown. There was no significant difference at all three time points between male (a) and female (b) groups ($P > 0·05$). (□), Natto; ▨, HU36; (■), Control.

Discussion

In the United States, bacteria considered safe for human consumption are assigned 'Generally Regarded As Safe' status (GRAS) by the Food and Drug Administration on a case-by-case basis. In the EU, a similar system is now under consideration referred to as 'Qualified Presumption of Safety' (QPS), whose aims are to harmonize the safety assessment of micro-organisms throughout the food chain (EFSA 2005; Anadon *et al.* 2006). This work provides a part of a long-term study on the safety of the members of the *Bacillus* genera for use as functional foods or probiotics, and supports a recent study of this type on the safety of two *Bacillus* species (*B. subtilis* and *Bacillus licheniformis*) contained in the probiotic Biosporin (Sorokulova *et al.* 2007).

Representatives of two spore-forming species, *B. subtilis* and *B. indicus*, were characterized for their safety as probiotic food supplements. In the case of *B. subtilis*, this species is a component of the Italian probiotic, Lactipan Plus [carrying a strain highly similar to PY79 (Hoa *et al.* 2000)], and in Japan, in the fermented soybean product Natto (*B. subtilis* var. *Natto*). In both cases, the number of viable *B. subtilis* present is greater than 10^{8}. At first inspection, *B. subtilis* should be safe for human consumption, yet no in-depth analysis of its safety has been

reported other than a recent study on the contents of Biosporin (Sorokulova *et al.* 2007). In the case of *B. indicus*, this species has not been used commercially but its ability to produce significant quantities of carotenoids could make it a suitable nutritional supplement, or 'nutrafood'.

Spores of all strains were shown to be completely resistance to exposure to SGF and SIF. This is important as it ensures that the stated dose actually reaches the region of the GIT where it should exert its effect (Hamilton-Miller and Gibson 1999). Having reached the small intestine, a proportion of spores should germinate in the nutrient-rich region of the jejunum and ileum where the pH of the gastric juices has been sufficiently reduced. We base this assumption on numerous molecular studies that have shown that *Bacillus* spores are capable of germinating in the jejunum and ileum (Hoa *et al.* 2001; Casula and Cutting 2002; Tam *et al.* 2006). Note that our results do not prove that HU36 spores can germinate, for which molecular analysis of vegetative mRNA is required. Persistence studies showed that for *B. subtilis* (PY79) and *B. indicus*, spores were no longer detectable in faeces after 14 days, which in the case of *B. subtilis* PY79, was in agreement with previous work (Duc *et al.* 2004). A pathogenic strain of *B. cereus* however persisted for a further 7 days, showing that it was better adapted to remain in the GIT. Biofilm-producing, undomesticated, strains of *B. subtilis* have been shown to remain longer in the GIT than domesticated strains, such as PY79 (Tam *et al.* 2006), and this attribute may enable them to persist longer compared with PY79 and HU36. In support of this, in other experiments, we have found that Natto, which forms biofilms, can survive within the mouse GIT for up to 19 days. Our study here also showed only Natto could sporulate anaerobically.

After spore germination, can the vegetative cell adhere to the intestinal tract? Adhesion to the mucus layer and the formation of biofilms are probably the most important elements in this process. *In vitro* adhesion assays determined that vegetative cells of HU36, Natto and PY79 had limited ability to bind to any of the cell lines, although HEp-2 binding was more significant for both *B. subtilis* strains. These results were generally in agreement with studies made on the adhesion of *Bacillus* species to Caco-2 and HEp-2 cells (Rowan *et al.* 2001). Interestingly, using these methods, we have found that it is not possible to measure adhesion of spores as we have found that they can germinate in cell culture medium.

Invasion was also measured, and it was surprising that in the case of Natto, significant levels of invasion to Caco-2 and HEp-2 cells could be observed and at levels greater than *L. monocytogenes* that was used as an invasive control. A low level of invasion was also observed with strain PY79. It is conceivable that the lipopeptide, surfactin, produced by Natto could facilitate entry into cells by rupturing the phospholipid membrane. On the other hand, our results showed the complete failure of any bacterial strain to invade HT29-16E cells which might result from the mucus layer secreted by these cells. Cytotoxicity assays showed that cell-free supernatants of Natto were able to lyse all three cell types. This activity however is unlikely to be surfactin as this lipopeptide is heat stable (From *et al.* 2007). The interference of low molecular weight compounds present in culture supernatants on *in vitro* cytotoxicity studies is recognized as a problem with *in vitro* testing (Rowan *et al.* 2001; From *et al.* 2007) and *in vivo* testing is preferred where appropriate (EFSA 2005).

An important aspect of safety assessments for potential microbial food supplements is to ensure that no enterotoxins are produced by the bacterium. Many strains of *B. cereus* are known to carry genes that encode one or more of four known enterotoxins (Hbl, Nhe, CytK and BceT) and one or more of these genes have been found on the chromosomes of other *Bacillus* species (Rowan *et al.* 2001; Guinebretiere *et al.* 2002). Both *B. subtilis* strains and *B. indicus* were shown to be devoid of known toxin genes. Other potential virulence factors found absent in these strains were haemolysis and the production of the phospholipase lecithinase, although the latter did give a weak reaction with *B. subtilis*, Natto.

Bacillus indicus was found to carry resistance to only one antibiotic, the macrolide clindamycin, whose target is the 23S rRNA preventing ribosome binding (Douthwaite 1992). The MIC was at a level above the EU-recommended breakpoint for this compound. In unpublished work (M. Urdaci; pers. comm.), 33 isolates of *Bacillus* strains have been tested of which over half showed resistance to clindamycin using the disc-diffusion method. Similarly, in a recent study of a *B. licheniformis* probiotic strain, a clindamycin MIC above the EFSA breakpoint was also identified (Sorokulova *et al.* 2007). Therefore, this may well be an intrinsic characteristic of *Bacillus* species and more work will be needed to demonstrate this. However, no plasmid could be recovered from HU36, and it seems unlikely that this resistance determinant could be transferred by conjugation. We also showed that HU36 did not appear to produce any antimicrobial activity using a panel of Gram-positive and Gram-negative bacteria. Although these studies are incomplete, a probiotic microbe that produces an antimicrobial related or similar to those used in human or veterinary medicine would be problematic and could contribute to the spread of antibiotic resistance and its use should be discouraged. In the case of Natto, clear activity against three indicator strains was seen. This activity is attributed to surfactin,

which is produced copiously by this particular strain of *B. subtilis* (Nagal *et al.* 1996).

In vivo studies are the preferred approach to substantiate *in vitro* analysis of safety (FAO/WHO 2002). For *B. subtilis*, safety in humans is already supported by the fact that *B. subtilis* is being consumed in traditional foods, such as Natto or as food supplements (as in Lactipan Plus in Italy). Traditional safety assessment techniques based on toxicological testing in animals may not be applicable in the case of most foods/novel foods, as this fact has been highlighted in a number of reports (e.g. FAO/WHO 2002). No specific guidelines are currently in place for assessing probiotics, but studies including acute (single-administration) toxicity and a repeated administration (chronic) toxicity assessment have been recommended (Ishibashi and Yamazaki 2001). One problem with the acute study is the maximum dose that can be physically administered to an animal preventing an accurate definition of the lethal dose $(LD)_{50}$. In any event, acute and chronic studies on *B. subtilis* and LAB have been reported (Zhou *et al.* 2000a,b; Sorokulova *et al.* 2007). Our data showed that for all three bacterial strains tested, there was no indication of pathogencity, infection or adverse effects.

In conclusion, as potential food supplements, *B. indicus* and *B. subtilis* appeared to show no sign of toxicity or virulence using *in vivo* assessments. The only issue regarding the use of *B. subtilis* and *B. indicus* is its intrinsic resistance to clindamycin, which may require a more detailed study.

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