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The Safety of Two *Bacillus* Probiotic Strains for Human Use

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Abstract Probiotics based on *Bacillus* strains have been increasingly proposed for prophylactic and therapeutic use against several gastro-intestinal diseases. We studied safety for two *Bacillus* strains included in a popular East European probiotic. *Bacillus subtilis* strain that was sensitive to all antibiotics listed by the European Food Safety Authority. *Bacillus licheniformis* strain was resistant to chloramphenicol and clindamycin. Both were non-hemolytic and did not produce Hbl or Nhe enterotoxins. No *bceT* and *cytK* toxin genes were found. Study of acute toxicity in BALB/c mice demonstrated no treatment-related deaths. The oral LD_{50} for both strains was more than 2×10^{11} CFU. Chronic toxicity studies were performed on mice, rabbits, and pigs and showed no signs of toxicity or histological changes in either organs or tissues. We demonstrated that while certain risks may exist for the *B. licheniformis* strain considering antibiotic resistance, *B. subtilis* strain may be considered as non-pathogenic and safe for human consumption.

Keywords Probiotics · *Bacillus* · Safety · Toxicity · Antimicrobials resistance

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Introduction

Health promoting microorganisms, e.g., probiotics, have been recently increasingly included in various food products and proposed for use as a food supplement or as a therapy for several infectious diseases [1, 2]. Probiotic therapy is very attractive because it is an effective and non-invasive low cost approach, which attempts to recreate natural flora rather than its disruption. Micro-organisms used as a probiotic for human are mainly gram-positive bacteria belonging to the *Lactobacillus* and *Bacillus* spp. For decades *Bacillus* bacteria and their metabolites have been used for several biotechnological applications, including enzymes, amino acids, and antibiotic production, preparation of fermented foods, and pest control. *Bacillus*-based preparations have been only more recentlyintroduced to the international market and are currently used as probiotics for bacteriotherapy and bacterioprophylaxis of gastrointestinal (GI) disorders in human [3–6].

Bacillus probiotics differ in many characteristics from those based on *Lactobacillus* spp. While lactobacilli represent a normal resident GI microflora of humans, the saprophytic bacteria of *Bacillus* genera belong only to the transitory GI bacteria. Thus, the use of *Bacillus* products raised a number of questions, including their safety.

Over the past 3 decades, this genus has expanded to accommodate more than 100 species (see www.dsmz.de/bactnom/nam0379.htm). However, only a few of these species are used as probiotics for human: *subtilis*, *licheniformis*, *clausii*, *coagulans*, *cereus*, *pumilus*, *laterosporus*, as well as some invalid species named as *toyoi* and *polyfermenticus* [7]. Although most of the species and genera are apparently safe, certain bacteria may be problematic, in particular, strains harboring transmissible antibiotic resistance determinants and bacilli that are known to produce

enterotoxins and/or an emetic toxin, belonging to the *B. cereus* group [8]. Several strains of *B. popilliae* and *B. pumilus* have been associated with endocarditis or cause symptoms mimicking listeriosis [9, 10]. The *B. subtilis* group species (*B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, and *B. pumilus*) have occasionally been reported to be associated with food poisoning. Both diarrheal and emetic types of outbreaks have been recorded, but very little is known about the nature of the toxins associated [11, 12]. Recently, WHO and the European Commission drew the attention to a number of scientific publications describing the detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. [13–16].

Therefore, the use of these bacteria in preparations for humans requires development of strict standards for safety control. Significant progress in legislation concerning this matter has been made in USA, Canada, and Europe [17–19]. However, no universal international standard for safety evaluation of these probiotics is available. In the US, bacteria considered safe for human consumption are awarded GRAS status ("Generally Regarded As Safe") by the Food and Drug Administration.

Recently, the European Food Safety Authority (EFSA) [19] proposed the introduction of the concept of "Qualified Presumption of Safety" (QPS), which could be applied to selected groups of microorganisms. The general considerations in a QPS scheme are as follows: (1) the importance of taxonomy in the risk assessment of micro-organisms, (2) familiarity of the use of the organism/s including its history of use for particular purposes, (3) pathogenicity (whether the grouping considered for QPS contains known pathogens, important in relation to *Bacillus* species and their toxigenic potential or to the virulent/avirulent forms of enterococci), (4) live bacteria directly consumed by humans or animals should be free of any acquired resistance to antibiotics which is of importance in both clinical and veterinary medicine, and (5) the absence of a capacity to produce antibiotics with structural similarities to those of importance in human and veterinary medicine likely to encourage development of resistance. The species traditionally included in the *B. subtilis* group can be considered as a group that agreed with QPS. However, due to the increased number of studies reporting the presence of toxins in several strains of *B. subtilis* group, it seems appropriate to analyze the safety of the potential probiotic strains from this bacterial group on an individual, strain-by-strain basis [19].

Very limited information is publicly available about the safety of *Bacillus* probiotics. In the present paper we evaluated the safety of two *Bacillus* strains: *B. subtilis* 3 (BS3) and *B. licheniformis* 31 (BL31), which are compounds of probiotic Biosporine® (Ukraine). This probiotic has a well-documented 10-year history of commercial application in Russia and the Ukraine for the efficient prevention and treatment of GI disorders [20–23]. Therefore, the aim of this study was to evaluate their safety according to the new requirement for probiotics.

Materials and methods

Bacterial strains and cultures

Bacillus subtilis VKPM B2335 (BS3) and *B. licheniformis* VKPM B2336 (BL31) were obtained from the Ukrainian Collection of Microorganisms (Kiev, Ukraine, http://www.imv.kiev.ua/Catalog/catalog.html). Strains were aerobically propagated in Mueller Hinton (MH) broth (Oxoid, Basingstoke, UK) or on MH agar for 48 h at 37°C. Bacterial cells were re-suspended in PBS buffer at three different concentrations (1×10^9, 1×10^{10}, 1×10^{12} CFU ml^{-1}). Bacteria recovered from commercial *Bacillus* probiotic preparations were used for comparative genotyping, i.e., *B. cereus* IP 5832 (Bactisubtil®, Cassenne Marion, Paris, France), *B. cereus* DM-423 (Cereobiogen®, Keda Drugs Trade Co. Ltd under Dalian University of Medical Sciences, China), *B. clausii* (Enterogermina®, Sanofi-Synthelabo, Milan, Italy), *B. cereus* (Biosubtyl®, Biophar Co. Ltd., DaLat, Vietnam). Strains *B. subtilis* PY79, a prototrophic strain of *B. subtilis* derived from the 168 type-strain [24], and *B. cereus* SC2329, a toxin-producing strain of *B. cereus* [25], were also used. All strains were aerobically propagated in MH broth (Oxoid, Basingstoke, UK) or on MH agar for 24 h at 37°C. For plasmid extraction experiments *Lactobacillus plantarum* strains were grown in Mann–Rogosa–Sharpe (MRS) broth media (BD Difco, Le Pont de Claix, France) for 8 h at 37°C.

Phenotypic characterization of *Bacillus* probiotic strains

Phenotypic characterization of *Bacillus* strains was done in accordance with the requirements laid out for the *Bacillus* genus [26]. An identification key proposed previously [27] was used for identification. This key is represented on-line at the following website http://www.imv.kiev.ua/key/.

16S rDNA sequencing

Genomic DNA from *Bacillus* strains was prepared as described previously [28]. The 16S rDNA gene fragments were amplified by PCR using the universal primers 20F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1500R (5′-GGTTACCTTGTTACGACTT-3′), which amplify the

maximum number of nucleotides in 16S rDNA from a wide variety of bacterial taxa [29]. PCR reaction and DNA sequencing were performed as previously described [28]. Databases (GenBank) were searched for sequences similarly to the 16S rDNA sequences obtained.

RAPD analysis

RAPD-PCR was realized according to Pinchuk et al. [30] by using two previously selected 10-mer primers (OPA3 and OPH8, Bioprobe, Montrevils-sous-Bois, France). A total of 25 µl of the PCR fingerprinting product was electrophoresed through an agarose gel, stained with ethidium bromide and photographed under UV light. A Lambda *Eco*RI/*Hin*dIII ladder was used as a size standard.

Plasmids analysis

Plasmid DNA was extracted from the *Bacillus* probiotic strains and from two *Lb. plantarum* strains (positive control strains) using the Voskuil and Chambliss method [31]. The samples were run at 10 V cm^{-1} in 1.0% agarose gels (1× TBE buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA) containing (0.25 mg ml^{-1}) ethidium bromide and photographed under UV light. A Lambda *Eco*RI/*Hin*dIII ladder was used as a size standard.

Antibiotic resistance analysis

Antibiograms for strains were realized by using the disc diffusion method according to the recommendations of the National Committee for Clinical Laboratory Standards [32] and the EFSA in 2005 [19]. Cells from the 18–24 h old cultures were suspended at approximately 1×10^8 CFU ml^{-1} (McFarland standard 0.5). This suspension was diluted 1:100 and 200 µl was seeded on MH agar plates using a swab. Antibiotic-impregnated discs (Biorad, Mitry Mory, France) were placed on seeded plates and the zone of growth inhibition was measured after 18 h of incubation at 37°C. Minimum inhibitory concentration (MIC) values were determined by using serial antibiotic dilution procedure in MH broth.

Enterotoxins and potential virulence factor analysis

Enterotoxin genes

Chromosomal DNA was isolated from strains and tested for the presence of *B. cereus* enterotoxin genes by using PCR as described previously to profile food-poisoning *Bacillus* strains [15, 33].

Enterotoxin detection

Enterotoxins were detected by using two commercial immunoassay kits. A BCET-RPLA kit (Oxoid) was used to detect the HblC subunit of the Hbl enterotoxin in enrichment cultures, while a Tecra BDE kit (Tecra Diagnostics) was used to detect the NheA subunit of the Nhe enterotoxin.

Hemolysis and lecithinase detection

Each strain was streaked on 5% sheep blood agar and *B. cereus* selective agar containing egg yolk and polymyxin B (Sigma) and incubated for 24–48 h at 37°C to detect patterns of hemolysis and lecithinase production, respectively.

Adhesion

Adhesion of *Bacillus* vegetative cells and spores to mucin and Caco-2 cells was performed according to the method described by Tallon et al. [34].

Toxicity study in vivo

Two hundred and twenty male BALB/c mice, 6–8 weeks of age, were housed (ten animals per cage). Sixty adult out bred male New Zealand white rabbits and 30 newborn piglets were kept in single cages. Animals were maintained in pathogen-free facility and housed in microisolator cages and had free access to standard laboratory chow and water. All animals were handled in accordance with Institutional Guidelines for the Care and Use of Laboratory Animals in Research and have been approved by the ethical committee of the French Agriculture Ministry for permission of animal experimentation (animal experiment grant no. 03640). The acute and chronic toxicity studies were performed in compliance with the EU general safety tests (Directive 92/18/EEC, part 7) and FDA GLP Regulations (9CFR113,64(b)) for the live bacterial vaccines.

For acute toxicity studies, mice were acclimatized under experimental conditions for 7 days

Then they were randomly assigned to 21 different groups of 10 mice each. Fresh bacterial cultures were grown on

MH agar for 48 h at 37°C and administrated intravenously (IV) and intraperitoneally (IP) at the levels of 5×10^7, 5×10^8, 5×10^9 CFU/mouse and orally at 5×10^7, 5×10^8, 2×10^{11} CFU/mouse. A total of 50 µl of cell suspensions was administrated at each inoculation. Mice of the control group were given sterile PBS. Animals were observed for 7 days. During this period, activity and behavior of each mouse were recorded daily. On days 2 and 7, five animals from each group were euthanized, and internal organs were observed macroscopically. For the groups treated orally, samples of different organs and tissues were collected for histological analysis: liver, kidneys, lungs, spleen, intestine, mesenteric lymph nodes, brain, thymus, and tissues around the throat.

Chronic toxicity studies were carried out using mice, rabbits, and piglets

A total of ten animals of each species (for each bacterial strain) were orally inoculated each day over a period of 10 days with bacterial cultures at doses of: mice, 1×10^6 CFU day^{-1}; rabbits and piglets, 1×10^9 CFU day^{-1}. Ten animals ofeach species in the placebo control group received sterile PBS. The treatment lasted 10 days, during which time the activity and behavior of each animal were observed. On day 11, all animals were euthanized, and internal organs were observed macroscopically. Samples of different organs and tissues were collected for histological analysis as described above.

In additional experiments, 20 rabbits (10 for each bacterial strain) were orally inoculated with bacterial cultures at a dose of 1×10^9 CFU day^{-1} for 30 days. Ten rabbits in the placebo control received sterile PBS. On day 31, samples of blood and different organs and tissues were collected.

Histology

Samples of organs and tissues were fixed in 10% formalin. Tissue sections were cut at 6 µm and stained with hematoxylin and eosin (H&E) standard stain as previously described [35].

Hematology

Blood samples were obtained from rabbits by cardiac puncture on day 10 and 30 after oral inoculation with bacteria. Blood analyses were made for each sample. Leukocytes were counted to determine the differential percentages of white blood cells (lymphocyte, monocytes, eosinophils, and heterophils). Total red blood cell (RBC), sedimentation rate and hemoglobin concentration were determined.

Statistics

The data were analyzed by using Student's *t*-test and the Fisher exact test. For all tests, the level of significance was set at $P < 0.05$. Unless otherwise indicated values in the text are means ± SEM.

Results

Phenotypic and molecular identification of two *Bacillus* probiotic strains BS3 and BS31

The phenotypic analysis of two *Bacillus* strains BS3 and BL31 demonstrated that these bacteria were gram-positive rods with the ability to form endospores under aerobic conditions and to produce catalase. Neither strain formed poly-β-hydroxybutyrate or produced egg-yolk lecithinase or hemolysins. The strains differed in their biochemical characteristics: strain BL31 (as opposed to strain BS3) grew anaerobically, produced arginine dihydrolase, formed gas from nitrate, and utilized propionate. These characteristics attributed to strain BS3 belong to *B. subtilis* and strain BL31 belongs to *B. licheniformis* species. Results were confirmed by 16S rDNA sequencing. Strain BS3 revealed 99.8% identity with *B. subtilis* 168 and strain BL31 revealed 99.9% identity with *B. licheniformis* ATCC14580.

The RAPD analysis has been used to differentiate BS3 and BL31 from other commercially available *Bacillus* probiotic strains. The OPA3 and the OPH8 primers selected previously for RAPD typing of *Bacillus* strains (30) allow the distinction between BS3 and BL31 from the four other commercial preparations containing *B. cereus* IP5832 (Bactisubtil), *B. cereus* DM-423 (Cereobiogen), *B. clausii* OC (Enterogermina), and *B. cereus* (Biosubtyl) (Fig. 1A).

Extraction of plasmids from the two *Bacillus* probiotic strains (BS3 and BL31) demonstrated that these strains did not harbor plasmids. In contrast, plasmids were isolated from the *Lb. plantarum* strains used as a positive control (Fig. 1B).

Antibiotic susceptibility of BS3 and BL31

The strains tested were found to be sensitive to most of the antibiotics used (Table 1). Strain BS3 was sensitive at all antibiotics listed by EFSA in 2005 [19]. In relation to the

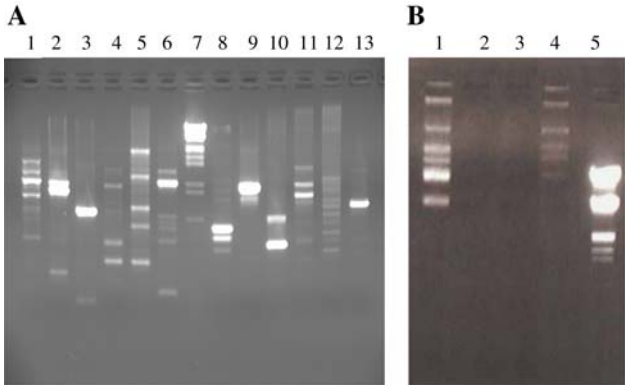

Fig. 1 RAPD and plasmid patterns of *Bacillus* strains from different probiotics. (**A**) RAPD profile of BS3 and BL31 was compared to other *Bacillus* probiotic strains. Lanes 1–6: OPA3 primer; line 7: λ *Eco*RI/*Hin*dIII ladder; lanes 8–12: OPH8 primer. Lanes 1, 8: BS3; lanes 2, 9: BL31; lanes 3, 10: *B. cereus* IP5832 (Bactisubtil); lanes 4,11: *B. cereus* DM-423 (Cereobiogen); lanes 5, 12: *B. clausii* (Enterogermina); lanes 6, 13: *B. cereus* (Biosubtyl). (**B**) Plasmid extraction analysis. Lane 1: *Lb. plantarum* R22; lane 2: strain BS3; lane 3: strain BL 31; lane 4: *Lb. plantarum* AFM16; lane 5: λ *Eco*RI/*Hin*dIII ladder

others antibiotics tested, BS3 was resistant to oxacillin and presented intermediate resistance to amoxicillin, methicillin, and some cephalosporins. Strain BL31 was resistant to two antibiotics listed by EFSA [19], chloramphenicol (with a MIC of 16) and clindamycin (with a MIC of 8). BL31 was also resistant or presented intermediate resistance to various antibiotics of the penicillin and cephalosporin family (Table 1).

Adhesion properties

The adhesion of probiotic *Bacillus* strains BS3 and BL31 to mucin and Caco-2 cells was studied. The probiotic strain *Lb. plantarum* 299v and a laboratory strain of *B. cereus* whose spores possess high adhesive properties were used as positive controls. We demonstrated that vegetative cells or spores of *B. subtilis* BS3 and *B. licheniformis* BL31 presented low adhesion properties for mucin (Table 2) and epithelial cell line Caco-2 (Table 3).

Evaluation of enterotoxins and potential virulence factor production by *Bacillus* probiotic strains

PCR was used to test chromosomal DNA from the *Bacillus* strains for the presence of enterotoxin genes as described previously by Duc et al. [36]. *Bacillus subtilis* BS3, *B. licheniformis* BL31, and the *B. subtilis* laboratory strain PY79 did not carry enterotoxin genes (Table 4). The *B. cereus* SC2329 strain was used as a positive control for all three genes (*nheA*, *nheB*, and *nheC*) encoding the non-hemolytic enterotoxin (Nhe). Similar observation has been made when the cultures of these strains have been tested for the production of the Nhe component using the ELISA test.

The tested *B. subtilis* strains BS3, BL31, and PY79 did not carry hemolysin genes. This is in contrast to *B. cereus* SC2329, which was found to carry three genes (*hblA*, *hblC*, and *hblD*) encoding hemolysin BL (Hbl). The Hbl is the primary virulence factor in *B. cereus*-associated diarrhea. Moreover, we failed to detect the Hbl enterotoxin in the two-probiotic strains BS3 and BL31 by using a Hbl enterotoxin reverse passive latex agglutination test kit (Table 4). The *bceT* gene, which encodes the single-component toxin enterotoxin T, was detected only in the *B. cereus* strain. No strain carried the *cytK* gene, which encodes the single-component toxin cytotoxin K.

Analysis of hemolytic capacity of tested strains demonstrated that only *B. cereus* strain produced complete hemolysis on sheep blood agar. The other strains were non-hemolytic (Table 4). We also tested for lecithinase production. *Bacillus cereus* was lecithinase positive, while all three *B. subtilis* strains were negative (Table 4).

Acute toxicity studies

Different doses of BS3 and BL31 were administrated orally (5×10^7 to 2×10^{11} CFU/mouse), IP and IV (5×10^7 to 5×10^9 CFU/mouse) to the BALB/c mice. There were no treatment-related deaths, even in groups of animals IP and IV treated with the *Bacillus* strains at the highest doses. Thus, the oral LD_{50} for the tested strains is more than 2×10^{11} CFU. LD_{50} for IP and IV-administrated BS3 and BL31 strains was more than 5×10^9 CFU. The administration of either BS3 or BL31 did not show any potential adverse effect on mouse activity and weight. All animals were clinically healthy, i.e., no diarrhea or other treatment-related illness was recorded.

There were no differences in the appearance of visceral organs between experimental and control groups of animals during macroscopic examination. On day 7 after the probiotic inoculations the spleen weight index (SWI) was measured for mice in the groups that were orally inoculated with the 5×10^9 CFU of *Bacillus* probiotic strains and compared to the placebo control (Table 5). No significant difference in SWI was observed between the groups. Microscopic observation found no signs of inflammation or any other pathological changes in all analyzed organs and tissues.

Table 1 Antibiotic resistance of *Bacillus* strains (disk and MIC methods)

Antibiotic (μg)[a]	Diameter of inhibition (mm)[b]		MIC (mg l^{-1})	MIC (mg l^{-1})	EFSA (2005) *Bacillus* break points, mg g^{-1}
	BS3	BL31	BS3	BL31	
Ampicillin (10)	20 ± 1 (S)	12 ± 1 (R)	16	16	n.r.[c]
Vancomycin (30)	21 ± 0.1 (S)	21 ± 0.5 (S)	0.5	0.5	4
Gentamycin (10)	27 ± 3 (S)	25 ± 1 (S)	<0.1	<0.1	4
Kanamycin (30)	22 ± 1.5 (S)	24 ± 0.5 (S)	1	4	8
Streptomycin (10)	21 ± 3 (S)	19 ± 3 (S)	4	4	8
Neomycin (30)	18 ± 0.5 (S)	19 ± 1.5 (S)	0.5	1	8
Erythromycin (15)	26 ± 0.5 (S)	24 ± 1 (S)	0.5	0.5	4
Clindamycin (2)	18 ± 1.5 (S)	7 ± 1 (R)	1	8	4
Quinupristin + dalfopristin	ND[d]	ND	4	4	4
Tetracyclin (30)	29 ± 0.5 (S)	27 ± 1 (S)	<0.1	4	8
Chloramphenicol (30)	18 ± 1 (I)	12 ± 1 (R)	8	16	8
Trimetoprim (5)	28 ± 0.5 (S)	30 ± 1 (S)	1	0.5	8
Linezolid (30)	28 ± 1 (S)	30 ± 3 (S)	ND[d]	ND[d]	4
Rifampicin (30)	20 ± 0.5 (S)	23 ± 0.6 (S)	<0.1	<0.1	
Ciprofloxacin (5)	30 ± 0.5 (S)	34 ± 0.5 (S)			
Enrofloxacin (5)	30 ± 3 (S)	32 ± 2 (S)			
Amoxicillin (30)	18 ± 0.4 (I)	16 ± 0.3 (I)			
Carbenicillin (100)	24 ± 0.6 (S)	18 ± 0.3 (S)			
Mezlocillin (75)	21 ± 0.8 (S)	17 ± 0.2 (I)			
Methicillin (5)	18 ± 0.1 (I)	6 (R)			
Oxacillin (1)	14 ± 0.3 (R)	6 (R)			
Ticarcillin (75)	24 ± 0.4 (S)	20 ± 0.1 (I)			
Imipenem (10)	36 ± 0.5 (S)	32 ± 0.3 (S)			
Cephalotin (30)	32 ± 0.5 (S)	20 ± 0.4 (S)			
Cefazolin (30)	24 ± 0.2 (S)	20 ± 0.1 (S)			
Cefamandol (30)	37 ± 0.6 (S)	16 ± 0.1 (I)			
Cefoxitin (30)	16 ± 0.3 (I)	12 ± 0.3 (R)			
Cefotaxim (30)	15 ± 0.2 (I)	10 ± 0.2 (R)			
Ceftriaxon (30)	18 ± 0.3 (I)	12 ± 0.2 (R)			
Amikacin (30)	20 ± 0.2 (S)	18 ± 0.1 (S)			
Tobramycin (10)	24 ± 0.3 (S)	20 ± 0.2 (S)			
Nitrofurantoin (300)	17 ± 0.2 (S)	18 ± 0.1 (S)			
Bactrim[e]	30 ± 0.4 (S)	30 ± 0.6 (S)			
Norfloxacin (10)	24 ± 0.2 (S)	24 ± 0.2 (S)			

[a] Antibiotic-impregnated discs (6 mm) with amount, in μg shown in brackets

[d] Diameter of inhibition from three individual experiments. (S): Sensible; (I): intermediate; (R): resistant

[c] Certain species inherently resistant

[d] Antibiotic or disks not possible for purchase

[e] Trimethoprim (1.25)/sulfamethoxazole (23.75)

Evaluation of *Bacillus* probiotic chronic toxicity

Chronic toxicity of probiotic strain BS3 and BL31 was studied in mice, rabbits, and pigs. There were no adverse effects on the general health status of the animals. No changes in the organs and tissues during histolopathological study were observed in treated animals. SWI of the mice in the probiotic treated groups was similar to that of the placebo control group (data not shown). There were no differences in the hematological indexes measured in the blood from control and treated rabbits (Table 6).

Table 2 Adhesion to mucin (CFU/well)

Species	Vegetative cells	Spores
Lb. plantarum 299v	12,536 ± 1,756	Nd[a]
B. cereus CH	1,600 ± 95	21,450 ± 3,120
B. subtilis 3	1	1,236 ± 120
B. lichenformis 31	1,700 ± 88	400 ± 36

[a] Nd (non-data): *Lactobacillus* not produces spores

Table 3 Adhesion to Caco-2 cells (bacteria/cell)

Species	Vegetative cells	Spores
Lb. plantarum 299v	7.54 ± 1.54	Nd[a]
B. cereus CH	2 ± 0.32	7.7 ± 1.62
B. subtilis 3	0.01	0.56 ± 0.06
B. lichenformis 31	0.1	0.5 ± 0.04

[a] Nd (non-data): *Lactobacillus* not producing spores

Discussion

Requirements for commercialization of probiotics are not fully defined for species that may cause disease in humans or harbor virulence factors. Examples of such bacteria are *Enterococcus* spp., *E. coli*, and some members of the *Bacillus* genus. While the pathogenic properties of *B. cereus* are well established, very limited information is available about risks associated with administration of other *Bacillus* species [37].

Absence of molecular information can lead to the incorrect identification of *Bacillus* probiotic strains [13, 25, 38, 39]. Therefore, according to the WHO and European Union regulations, the data on probiotic strain safety must include the phenotypic and genotypic characterization including 16S rRNA sequencing [19, 40, 41]. In the present study we confirmed that the probiotic strain BS3 is indeed a strain of *B. subtilis* and the strain BL31 is a strain of *B. licheniformis* species by using phenotypic characterization and 16S rDNA gene sequencing. Using RAPD OPA3 and OPH8 primers we were able to distinguish BS3 and BL31 from each other and from other tested *Bacillus* probiotic strains. Thus, these primers could be useful in the purity control of these strains during probiotic production and manufacturing.

Table 5 Spleen weight index (SWI)[a] of mice inoculated orally with bacterial strains

Groups of mice[b]	Number of mice in group	SWI, mg g^{-1}
BS3	10	3.41 ± 0.18
BL31	10	3.37 ± 0.16
Control	10	3.40 ± 0.14

[a] SWI = spleen weight (mg)/mouse body weight (g)

[b] BALB/c mice were orally inoculated with the 5×10^9 CFU of *Bacillus* probiotic strains or the placebo control as indicated in Materials and Methods. On day 7 after inoculation the SWI was measured

Antibiotic susceptibility tests indicate that strain BS3 was sensitive to all antibiotics included in the EFSA 2005 list [19]. Strain BL31 was resistant to chloramphenicol (MIC: 16 mg l^{-1}) and to clindamycin (MIC: 8 mg l^{-1}). We have previously tested 33 isolates of *Bacillus* strains and more than half of them were resistant to clindamycin using the disc-diffusion method (data not shown). Resistance to a given antibiotic can be inherent to a bacterial species or genus. Therefore, resistance to clindamycin may be an intrinsic characteristic of *B. licheniformis* species and might be useful for the *Bacillus* taxonomy as has been

Table 4 Potential virulence traits of commercial strains

Strain	*Bacillus* species	Hem[a]	Lec[b]	HBL complex[c]				Oxoid kit test index[d]	NHE complex[c]			Tecra kit test index[e]	*cytK*[c]	*bceT*[c]
				hblA	*hblB*	*hblC*	*hblD*		*nheA*	*nheB*	*nheC*			
PY79	*B. subtilis*	γ	–	–	–	–	–	0	–	–	–	1	–	–
BS3	*B. subtilis*	γ	–	–	–	–	–	0	–	–	–	1	–	–
BL31	*B. licheniformis*	γ	–	–	–	–	–	0	–	–	–	1	–	–
SC2329	*B. cereus*	α	+	+	–	+	+	128	+	+	+	4	–	+

[a] Hemolysis; α, brownish zone around colonies due to loss of potassium in red cells; β, complete hemolysis, clear zone around colony; γ, no change

[b] Lecithinase; +, blue precipitation of hydrolyzed lecithin around peacock blue colonies (typical for *B. cereus*); –, no changes

[c] +, a PCR product of the expected size was observed; –, no PCR product was observed

[d] For the Oxoid test, the indices corresponded to the last supernatant dilution (in two-fold serial dilutions) for which enterotoxin remained detectable. According to the manufacturer's instruction, strains with an index of 0 were considered negative, and the sensitivity of the test is 2 ng ml^{-1}

[e] For the Tecra test, indices from 1 to 5 corresponded to the coloration intensity. According to the manufacturer's instruction, strains with an index of <3 were considered negative, and the sensitivity of the test is 1 ng ml^{-1}

Table 6 Hematology measurements (mean ± SEM) of rabbits orally administered with 1×10^9 CFU of *Bacillus* strains or placebo control for 30 days

	Control[b]	BS3	BL31
Sedimentation rate (mm h^{-1})	1–2	1–2	1–2
Hemoglobin (g l^{-1})	123.80 ± 6.20	130.33 ± 7.30	127.50 ± 6.80
RBC[a] count ($\times 10^{12}$ l^{-1})	5.30 ± 1.80	5.60 ± 1.50	5.50 ± 1.40
Leukocytes count ($\times 10^9$ l^{-1})	7.80 ± 0.80	7.40 ± 0.60	7.20 ± 0.90
Heterophils (%)	43.27 ± 3.70	43.52 ± 3.69	42.62 ± 3.39
Lymphocytes (%)	49.40 ± 2.07	48.90 ± 2.91	49.89 ± 1.26
Monocytes (%)	3.60 ± 0.90	3.39 ± 0.80	3.62 ± 0.90
Eosinophils (%)	3.73 ± 1.30	4.19 ± 1.20	3.87 ± 1.30

[a] RBC: red blood cells

[b] Rabbits, which obtained PBS placebo control

previously discussed [42, 43]. We also determined that BS3 and BL31 strains do not carry plasmids. This finding is very important, since antibiotic resistance plasmids are of special interest from the safety point of view, because they may be conjugatively transferred to other strains. Concerning strain BL31, complementary studies are necessary in order to exclude that chloramphenicol and clindamycin resistances are harbored in a transposable element.

It has been proposed that production of antimicrobial substances by probiotics, such as bacteriocins and antibiotics, is one of the mechanisms by which they protect the host against pathogenic microorganisms [7]. However, according to EFSA 2005 [19] requirements, these antibiotics should be different from those used for human or veterinary medicine. We previously demonstrated that strain BS3 produces a heat-stable, protease-resistant antimicrobial substance that inhibits growth of many bacteria including *S. aureus*, *Helicobacter*, and *Campylobacter* spp. [44]. The compound, purified from the cell-free supernatant, was identified as amicoumacin A, an isocoumarin antibiotic with anti-inflammatory properties, not used in human and veterinary medicine. In contrast to BS3 strain BL31 presents a weak and very limited antimicrobial activity [7].

The risk of food-borne diseases due to *Bacillus* spp. other than *B. cereus* has been considered low, and the occasional presence of *B. cereus* toxins in strains belonging to several *Bacillus* spp. has been reported [8, 12, 15, 16]. By using PCR analysis we demonstrated that BS3 and BL31 do not carry enterotoxin operon Hbl and Nhe. The absence of the toxin production was confirmed by using Oxoid RPLA and Tecra BDE kits. Genes for the toxins *bceT* and *cytK* were also absent. Our data are in accordance with the report of From et al. [14] in which 333 *Bacillus* strains outside the *B. cereus* group has been analyzed, and none of these strains produced *B. cereus*-like toxins. Therefore, it is not clear if *Bacillus* strains outside the *B. cereus* group may produce the same enterotoxins as those of *B. cereus*, or if other enterotoxins are involved.

It is important to note that strong hemolytic and/or lecithinase activity might be an indication of the presence of cytotoxic phopholipases that are associated with virulence of a given bacterial strain. Our data indicate that BS3 and BL31 do not produce lecithinase and do not have hemolytic activity. Therefore, the absence of these activities combined with the absence of *B. cereus*-like toxins suggests that BS3 and BL31 do not present a risk for human health. The in vivo study of acute and chronic toxicity in animal models remains the most accurate mechanism to identify the major effects of probiotics on the host. In order to complete the study of toxicity of BS3 and BL31 strains we undertook an in vivo study of acute and chronic toxicity. Although acute toxicity tests were originally designed for chemicals, they also give an indication of any harmful effects associated with extremely high doses of live bacteria. To study acute toxicity we inoculated mice with 10 and 100 times the recommended dose of bacteria. There were no treatment-related deaths or illness, even using the highest doses. Thus, the oral LD_{50} for the tested strains is more than 2×10^{11} CFU. The same values were obtained for the probiotic strains of *Lactobacillus* [45, 46]. The LD_{50} for intraperitoneal and intravenous administration of BS3 and BL31 strains was more than 5×10^9 CFU, whereas administration of *B. cereus* IP5832 from the probiotic Bactisubtil at the same dose was 100% lethal [47].

Chronic toxicity of BS3 and BL31 strains was studied for three species of animals, mice, rabbits, and pigs. No adverse effects were detected. Obtained results are in accordance with the data for *Lactobacillus* probiotics [46].

Although the presence or absence of adhesins per se is not useful to define a safe organism, we tested adhesion of BS3 and BS31 strains to mucin and Caco-2 cells. Strains were found to be poor adherents indicating improbable

invasiveness. These results were confirmed in our in vivo study in mice demonstrating the clearance of the BS3 strain 2 days after administration of the last oral dose (using a dose of 5×10^9 CFU every 2 days for 1 week, data not shown).

Taken together our data indicate that both *Bacillus* strains tested in this study were sensitive to the antibiotics listed in EFSA (excepted chloramphenicol and clindamycin to BL31), non-toxinogenic and presented no toxicity in vivo. According to these data strain BS3 may therefore be considered as non-pathogenic and safe for human consumption, while certain risks may exist for BL31. A complementary antibiotic resistance study of BL31 is necessary in order to conclude the safety of it use. In conclusion, the frequency of the studies recommending the administration of probiotics for the treatment of critically ill patients has increased in the last decade. Therefore, data presented herein point out that an evaluation of the safety of new or exciting *Bacillus* probiotics is highly recommended prior to their use for humans.

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