Food Microbiology 28 (2011) 214–220

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Review

Bacillus probiotics

Simon M. Cutting*

School of Biological Sciences, Royal Holloway University of London, Egham, Surrey TW20 0EX, UK

ARTICLE INFO

Article history:
Available online 24 March 2010

Keywords:
Bacillus
Spores
Probiotics
Feed supplements

ABSTRACT

Bacterial spore formers are being used as probiotic supplements for use in animal feeds, for human dietary supplements as well as in registered medicines. Their heat stability and ability to survive the gastric barrier makes them attractive as food additives and this use is now being taken forward. While often considered soil organisms this conception is misplaced and Bacilli should be considered as gut commensals. This review summarises the current use of *Bacillus* species as probiotics, their safety, mode of action as well as their commercial applications.

1. Bacterial spores

Bacterial spores are produced in nature as a means to survive extreme environmental conditions enabling long-term survival in conditions that could otherwise kill vegetative bacteria (Nicholson et al., 2000). The decision to sporulate is very much dependant upon the decline in nutrients in the immediate vicinity of the live cell. Sensing this, the bacterium enters an irreversible program of development that results in the production of a spore some 8 h later (Fig. 1) (Errington, 2003). Intrinsic to survival is the structure of the bacterial endospore, that contains, at its core, a condensed and inactive chromosome. Additional layers surround the spore, including a peptidoglycan-rich cortex and one or more layers of proteinaceous material referred to as the spore coat (Henriques and Moran, 2007). Together these protect the spore from UV radiation, extremes of heat (typically up to 80–85 °C in most species), exposure to solvents, hydrogen peroxide and enzymes such as lysozyme (Nicholson et al., 2000). The spore itself, is dehydrated and if exposed to appropriate nutrients will germinate, a process taking just a few minutes, allowing water to enter the spore, breakage and removal of the spore coats, and outgrowth and resumption of vegetative cell growth (Fig. 1) (Moir, 2006). Depending on species spores are spherical or ellipsoidal in shape, between 0.8 and 1.4 mm in length, have a negative surface charge and are moderately hydrophobic. Spore forming bacteria commonly fall under two genera, *Bacillus* and the strictly anaerobic *Clostridia* although a surprisingly large number of other, lesser-known, genera include spore formers.

2. The use of *Bacillus* as probiotics

Probiotics are live microbes, which when administered in adequate amounts confer a health benefit to the host (Araya et al., 2002). *Bacillus* species have been used as probiotics for at least 50 years with the Italian product known as Enterogermina® registered 1958 in Italy as an OTC medicinal supplement. The scientific interest in *Bacillus* species as probiotics though, has only occurred in the last 15 years and three principal reviews have covered the field (Hong et al., 2005; Mazza, 1994; Sanders et al., 2003). Of the species that have been most extensively examined these are *Bacillus subtilis*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans* and *Bacillus licheniformis*. Spores being heat-stable have a number of advantages over other non-spore formers such as *Lactobacillus* spp., namely, that the product can be stored at room temperature in a desiccated form without any deleterious effect on viability. A second advantage is that the spore is capable of surviving the low pH of the gastric barrier (Barbosa et al., 2005; Spinosa et al., 2000) which is not the case for all species of *Lactobacillus* (Tuohy et al., 2007) so in principle a specified dose of spores can be stored indefinitely without refrigeration and the entire dose of ingested bacteria will reach the small intestine intact.

Spore probiotics are being used extensively in humans as dietary supplements (Table 1), in animals as growth promoters and competitive exclusion agents (Table 2) and lastly in aquaculture for enhancing the growth and disease-resistance of cultured shrimps, most notably the Black Tiger shrimp (*Penaeus monodon*) (Table 3). This review will focus primarily on the use of spore products for human use. Interestingly, a number of *Bacillus* products are licensed as medicinal supplements. Rather than describing specific products a short summary of the major *Bacillus* species used in commercial products will be summarised.

* Tel.: +44 (0) 1784 443760; fax: +44 (0) 1784 414224.
E-mail address: s.cutting@rhul.ac.uk

0740-0020/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2010.03.007

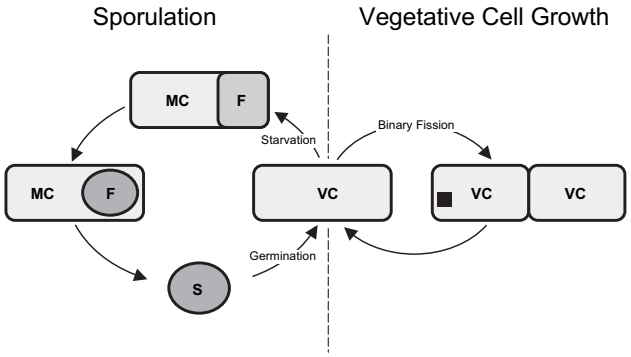

Fig. 1. The sporulation life cycle. A schematic showing the opposed life cycles of bacterial spore formers. Under conditions of nutrient starvation the growing, vegetative cell (VC) will undergo a series of morphological changes that create a forespore (F) within the mother cell (MC) of the sporangium. After approximately 8 h the spore (S) is released by lysis of the MC.

2.1. *B. clausii*

B. clausii spores are used in the product Enterogermina® which is registered as an OTC medicinal supplement. Unlike most probiotic formulations that are supplied in tablet or capsule form the Enterogermina product carries, spores (2×10^9) suspended in 5 ml of water and 2–3 vials are taken each day with the aim of preventing infantile diarrhoea (Figs. 2–4). The suspension of spores in water is thought to enhance delivery of spores to the mucosa and demonstrates the versatility of spore formulations. The product carries four antibiotic resistant strains of *B. clausii* that are recommended for use with antibiotics (Coppi et al., 1985; Green et al., 1999; Senesi et al., 2001). The four strains are each derived from ATCC 9799, a penicillin-resistant strain originally designated as *B. subtilis*. Through a multi-step process strains resistant to novobiocin + rifampin (strain N/R), chloramphenicol (strain O/C), streptomycin + neomycin (strain SIN) and tetracycline (strain T) have been obtained (Ciffo, 1984; Mazza, 1994). Interestingly, these *B. clausii* strains also carry resistance to a number of other antibiotics including erythromycin, cephalosproins and cycloserine, kanamycin, tobramycin, and amikacin (Mazza et al., 1992). It has now been demonstrated that the resistance genes within these *B. clausii* strains are stable and are unable to transfer (Bozdogan et al., 2004; Mazza, 1983; Mazza et al., 1992).

Although the initial scientific studies used to register this product in 1958 are obscure clinical trials have subsequently been performed demonstrating efficacy, although a number of these trials lack completeness in terms of controls. Of note are clinical studies assessing the effect of Enterogermina modulating the immune responses in allergic children with recurrent respiratory infections (Ciprandi et al., 2004, 2005a,b). After administration of the probiotic nasal symptoms and eosinophil counts in allergic

Table 1
Bacillus probiotics for human use.[a]

Product	Manufacturer	Comments/references
Bactisubtil®	Produced by Marion Merrell (Levallois-Perret, France) but also by Hoechst and then Aventis Pharma following merger acquisitions. Also cited as being produced by Casella-Med, Cologne, Germany	Capsule carrying 1×10^9 spores of *Bacillus cereus* strain IP5832[b] (ATCC 14893) [n.b., originally deposited as *B. subtilis*.
Bio-Kult®	Protexin Health Care http://www.bio-kult.com	*B. subtilis* is one component of 14 strains carried in this UK probiotic supplement.
Biosporin®	(1) Biofarm, Dniepropetrovsk, Ukraine	Biosporin® is a mixture of two strains of living antagonistic bacteria *B. subtilis* 2335 (sometimes referred to as *B. subtilis 3*) and *B. licheniformis 2336* (ratio is 3:1). Originally isolated from animal fodder.
	(2) Garars, Russia	There are a number of versions of this products produced in different countries including a recombinant form, Subalin.
Biovicerin®	Geyer Medicamentos S.A. Porto Alegre, RS, Brazil http://www.geyermed.com	*B. cereus* strain GM Suspension of 10^6 spores ml^{-1}.
Bispan®	Binex Co. Ltd., Busan, S. Korea www.bi-nex.com	Tablet carrying spores (1.7×10^7) of *B. polyfermenticus* SCD.[c]
Domuvar	BioProgress SpA, Anagni, Italy http://www.giofil.it	Vial carrying 1×10^9 spores of *Bacillus clausii* in suspension, labelled as carrying *B. subtilis*. No longer marketed.
Enterogermina®	Sanofi Winthrop SpA, Milan, Italy www.automedicazione.it	Vial (5 ml) carrying 1×10^6 spores of *B. clausii* in suspension. At least four different strains of *B. clausii* present and product originally labelled as carrying *B. subtilis*.
Flora-Balance	Flora-Balance, Montana, USA www.flora-balance.com	Capsules labelled as carrying *Bacillus laterosporus* BOD[c] but containing *Brevobacillus laterosporus* BOD.
Sustenex®	Ganeden Biotech Inc., Ohio, USA www.sustenex.com	*B. coagulans* GanedenBC[30] This is a patented strain that has GRAS approval in the USA.
Lactipan Plus	Istituto Biochimico Italiano SpA, Milan, Italy	Capsule carrying spores of *B. subtilis* labelled as carrying 2×10^9 spores of *Lactobacillus sporogenes*.[c]
Lactospore	Sabinsa Corp., Piscataway, NJ, USA www.sabinsa.com	Labelled as *Lactobacillus sporogenes*[c] but contains *B. coagulans* $6–15 \times 10^9$ g^{-1}.
Medilac-Vita	Hanmi Pharmaceutical Co. Ltd., Beijing, China http://www.hanmi.co.kr	*B. subtilis* strain RO179 (at 10^8 g^{-1}) in combination with *Enterococcus faecium*.
Nature's First Food	Nature's First Law, San Diego, CA, USA http://www.rawfood.com	42 species listed as probiotics including: *B. subtilis*, *B. polymyxa*,[c] *B. pumilus* and *B. laterosporus*.[c]
Neolactoflorene	Newpharma S.r.l., Milan, Italy	Mixture of lactic acid bacteria inc. *L. acidophilus*, *B. bifidum* and *L. sporogenes*.[c] *L. sporogenes* at 3.3×10^5 CFU g^{-1} whose valid name *is B. coagulans* and is mislabelled as a strain of *B. subtilis*.
Primal Defense™	Garden of Life® Palm Beach, Florida, USA. www.gardenoflife.com/	*B. subtilis*.

[a] This list is likely incomplete and excludes Vietnamese products that are shown in Table 4.
[b] Contains the same strain used in the now discontinued animal feed product Paciflor.
[c] Not recognised as a *Bacillus* species (www.bacterio.cict.fr).

Table 2
Bacillus probiotics for veterinary use.[a]

Brand	Animal	Manufacturer	Comments
AlCare™	Swine	Alpharma Inc., Melbourne, Australia www.alpharma.com.au/alcare.htm	*B. licheniformis* (NCTC 13123) at 10^9–10^{10} spores kg^{-1}. This is a non-bacitracin producing strain. Not licensed in the EU.
BioGrow®	Poultry, calves and swine	Provita Eurotech Ltd., Omagh, Northern Ireland, UK http://www.provita.co.uk	Listed as containing spores of *B. licheniformis* (1.6×10^9 CFU g^{-1}) and *B. subtilis* (1.6×10^9 CFU g^{-1}).
BioPlus® 2B	Piglets,[a] Chickens, turkeys for fattening[c]	Christian Hansen Hoersholm, Denmark http://www.chbiosystems.com	Mixture (1/1) of *B. licheniformis* (DSM 5749) and *B. subtilis* (DSM 5750) at 1.6×10^9 CFU g^{-1} of each bacterium. EU approved.[a]
Esporafeed Plus®	Swine	Norel, S.A. Madrid, Spain	1×10^9 *B. cereus* (CECT 953). Not licensed in the EU.
Lactopure	Poultry, calves and swine	Pharmed Medicare, Bangalore, India http://www.pharmedmedicare.com	Labelled as *Lactobacillus sporogenes*[b] but contains *B. coagulans*.
Neoferm BS 10	Poultry, calves and swine	Sanofi Sante Nutrition Animale, France	2 strains of *B. clausii* (CNCM MA23/3V and CNCM MA66/4M). Not licensed in the EU.
Toyocerin®	Calves, poultry, rabbits and swine. Possible use also for aquaculture	Asahi Vet S.A., Tokyo (Head Off.), Japan http://www.asahi-kasei.co.jp	*B. cereus* var. toyoi (NCIMB-40112/CNCM-1012) at a minimum concentration of 1×10^{10} CFU g^{-1} mixed with maize flour (4% by weight) and calcium carbonate (90% by weight). Licensed in the EU.[a]

[a] Authorised for unlimited use by the EU.
[b] Not recognised as a *Bacillus* species (www.bacterio.cict.fr).

children were significantly reduced. In these studies a Th1 (T-helper 1) bias was observed showing that ingestion of Enterogermina could enhance the cellular immunity in allergic children who normally carry a Th2 bias. These studies have been supported by later studies by Marseglia et al. (2007) who have examined the duration and rate of respiratory infections in 40 children (mean age 4.3 ± 1.5 years). After administration of Enterogermina for 90 days they observed a decrease in the duration of respiratory infection, but not the frequency of infection. Other clinical trials have examined the positive effect of Enterogermina on the side effects of antibiotic-based *Helicobacter pylori* therapy (Nista et al., 2004), and on urinary tract infections (Fiorini et al., 1985).

The product was originally labelled as carrying spores of *B. subtilis* but subsequent studies have identified the species as *B. clausii* (Green et al., 1999; Senesi et al., 2001). This product is not specifically referred to as a probiotic but claims to enhance the body's immune system following germination of the spores in the small intestine.

2.2. B. coagulans

This species is often labelled, incorrectly, as *Lactobacillus sporogenes* which is an unrecognised species name. The origin of this species for use in probiotics stems from India where a number of manufacturers produce *B. coagulans* as a food ingredient for export and relabelling in Europe and the US. *B. coagulans* secretes a bacteriocin, Coagulin, which has activity against a broad spectrum of enteric microbes (Hyronimus et al., 1998). Recently one strain, labelled as GanedenBC^{30} has been granted self-affirmed GRAS status by the FDA in the US. Marketed by Ganeden, as GanedenBC^{30} it is being used in a number of products such as Sustenex and is also being incorporated into foods where spores can survive the mild heat-treatments used to sterilise foods. A recently published randomized, double-blind, placebo-controlled, parallel-design, has shown significant effects of *B. coagulans* as an adjunct therapy for relieving symptoms of rheumatoid arthritis (Mandel et al., 2010). Other than this the value of *B. coagulans* as a probiotic has, however, recently been questioned (Drago and De Vecchi, 2009) and undoubtedly, further scientific evidence supporting the efficacy of this species is required.

2.3. B. subtilis and B. licheniformis

B. subtilis has been extensively studied at a genetic and physiological level. Numerous probiotic products are labelled as carrying *B. subtilis* and in part, this probably results historically from a carelessness in assuming that most aerobic spore formers are *B. subtilis*. Accordingly, numerous products claiming to carry *B. subtilis* have been shown to carry other species (see Table 1 and Table 4). However, *B. subtilis* var. Natto is worthy of comment. This bacterium is used in the fermentation of soybeans that is used to prepare the Japanese staple known as Natto. Natto carries as many as 10^8 viable spores per gram of product and for decades health benefits have been associated with consumption of Natto including stimulation of the immune system (Hosoi and Kiuchi, 2004). A serine protease known as Nattokinase is secreted from vegetative cells of *B. subtilis* var. Natto and has been shown to reduce blood clotting by fibrinolysis (Sumi et al., 1987, 1995). There are several important points here, firstly, the serine protease that is named Nattokinase is in fact produced by all strains of *B. subtilis* but in the

Table 3
Bacillus probiotics for aquaculture.[a]

Brand	Manufacturer	Comments
BaoZyme-Aqua	Sino-Aqua Corp., Kaohsiung, Taiwan www.sino-aqua.com	*B. subtilis* strains Wu-S and Wu-T at 10^8 CFU g^{-1}, product also contains *Lactobacillus* and *Saccharomyces* spp.
Biostart®	Microbial Solutions, Johannesburg, South Africa and Advanced Microbial Systems, Shakopee, MN, USA	Mixture of: *B. megaterium*, *B. licheniformis*, *Paenibacillus polymyxa* and two strains of *B. subtilis*.
Liqualife®	Cargill, Animal Nutrition Division www.cargill.com	Undefined *Bacillus* species.
Promarine®	Sino-Aqua company Kaohsiung, Taiwan www.sino-aqua.com	Carries four strains of *B. subtilis*.
Sanocare Sanolife Sanoguard	INVE Technologies nv Dendermonde, Belgium www.inve.com	Various *Bacillus* species.

[a] This shows just a selection of registered products from international companies. In shrimp-producing countries the number of 'local' products is substantial, for example, in Vietnam over 30 different products are sold.

Fig. 2. Enterogermina®. This is a licensed OTC product containing 2×10^9 of GMP-produced spores of *B. clausii* in 5 ml of water. 2–3 vials are consumed per day to help prevent gastroenteritis in infants and children.

Natto strain it is produced at high levels. Second, it cannot be ruled out that health benefits ascribed to Natto require consumption of both soybeans and bacteria, rather than just the bacterium. In any event, Nattokinase has GRAS status as an enzyme produced from a bacterium in the US and is purified and sold as a health supplement worldwide. In poultry studies controlled trials have shown that oral administration of *B. subtilis* spores reduce infection by *Salmonella enterica* serotype Enteritidis, *Clostridium perfringens* and *Escherichia coli O78:K80* (La Ragione et al., 2001; La Ragione and Woodward, 2003).

B. subtilis and *B. licheniformis* are used together in two products, Biosporin and BioPlus® 2B. BioPlus® 2B is used in animal feed while Biosporin is licensed as a medicine in the Ukraine and Russia. Biosporin is sold in glass vials that must be reconstituted in water before consumption. The two *Bacillus* strains, *B. subtilis 2335* and *B. licheniformis 2336* are well characterised and a number of clinical studies have been used to demonstrate probiotic effects although none been performed with the rigour of a full clinical trial (Bilev, 2002; Osipova et al., 2003, 2005; Sorokulova, 1997; Sorokulova et al., 1997). Interestingly, *B. subtilis 2335* has been shown to produce the antibiotic Amicoumacin with *in vitro* activity against

Fig. 3. Biosubtyl and Biosubtyl DL. Typical Vietnamese products, in this case, Biosubtyl that carries spores of *B. cereus* IP5832 and Biosubtyl DL carrying a mixture of *B. cereus* IP5832 and *Lactobacillus acidophilus*. Neither product is labelled properly nor carries the stated dose.

Fig. 4. Natto. Natto is normally consumed as a fermented soybean product either hot or cold. In this example it is sold as a snack with dried soybeans coated with a fine white powder of *B. subtilis* var. Natto, the active ingredient required for the taste and texture of Natto.

H. pylori (Pinchuk et al., 2001). In the case of BioPlus® 2B this animal feed product has also been extensively studied with numerous efficacy studies focused on the suppression of gastrointestinal pathogens completed resulting in the registration of this product as a feed supplement in Europe (SCAN, 2000b). It remains unclear whether there is any added benefit in the combined use of the two species.

2.4. *B. cereus*

B. cereus is a known human pathogen that is the cause of mild food poisoning due to the production of up to three enterotoxins and one emetic toxin (Stenfors Arnesen et al., 2008). Not all strains of *B. cereus* carry enterotoxin genes yet a number of *B. cereus* probiotics have been shown to carry the enterotoxin genes (Hoa et al., 2000) and one product, Paciflor, used in animal feed has been withdrawn from use in the EU (SCAN, 2001a). Despite this *B. cereus* products are still being used for example, Toyerocin®, an animal feed product is registered for use in Europe (SCAN, 2001b) and Bactisubtil® as a registered medicinal supplement for human use. Interestingly, the strain of *B. cereus* used in Bactisubtil® known as IP5832 is the same as that in the withdrawn animal product Paciflor®.

3. How do spore probiotics work?

Bacillus species are often considered soil organisms since spores they can readily be retrieved from soil. However, attempting to isolate vegetative bacteria from soil is more problematic and it now

Table 4
Vietnamese *Bacillus* OTC products licensed for human use.

Brand	Manufacturer	Comments
Bio-Acimin	Viet-Duc Pharmaceutical Co. Ltd., Hanoi	Labelled as containing *B. subtilis*, *L. acidophilus*, *S. faecalis* but *B. subtilis* is *B. cereus* at 10^7 g^{-1}.
Bibactyl	Tediphar Corporation (TEDIPHARCO), Ho Chi Minh City, Vietnam	Sachet (1 g) carrying 10^7–10^8 spores of *B. subtilis*.
Bidisubtilis	Bidiphar. Binh Dinh Pharmaceutical and Medical Equipment Company, 498 Nguyen Thai Hoc, Qui Nhon, Vietnam	Labelled sachets carrying 1×10^6 spores of *B. cereus* but mislabelled as *B. subtilis*.
Biosubtyl	Biophar Company, Da lat, Vietnam	Sachet (1 g) carrying 10^6–10^7 of *B. cereus* spores mixed with tapioca. Product labelled as *B. subtilis*. The strain is closely related by 16S rRNA analysis to IP 5832 used in Bactisubtil®.
Biosubtyl DL	IVAC, 18 Le Hong Phong, Da Lat, Vietnam	Sachets (1 g) carrying 10^7–10^8 CFU of *B. subtilis* and *Lactobacillus acidophilus*.
Biosubtyl I and II	Biophar Company, Nha Trang, Vietnam	Sachet (1 g) carrying 10^6–10^7 of *B. pumilus* spores mixed with tapioca. Product labelled as *B. subtilis*.
Pastylbio	Pasteur Institute of Ho Chi Minh City, Vietnam	Sachets (1 g) carrying 10^8 spores of *B. subtilis*.
Subtyl	Mekophar, Pharmaceutical Factory No. 24, Ho Chi Minh City, Vietnam	Capsule carrying 10^6–10^7 spores of a *B. cereus* species termed *B. cereus* var. vietnami. Product labelled as carrying *B. subtilis*.
Biobaby	ILdong Pharm Co., Ltd., 60-1, SinKeonji-Dong, Ansung-Si, Kyong Ki-Do, Korea	Each gram of granules contains: *Lactobacillus sporogenes* 5.0×10^7 cfu *Clostridium butyricum* 1.0×10^7 cfu *Bacillus subtilis* 3.0×10^6 Thiamine Nitrate 0.3 mg Riboflavin 0.2 mg Ascorbic Acid 5.0 mg Nicotinamide 0.1 mg Dibasic calcium photphate 20.0 mg Dried yeast 50.0 mg
Ildong Biovita	ILdong Pharm Co., Ltd., 60-1, SinKeonji-Dong, Ansung-Si, Kyong Ki-Do, Korea	Each gram of granules contains: *Lactobacillus sporogenes* 5.0×10^7 cfu *Clostridium butyricum* 1.0×10^7 cfu *Bacillus subtilis* 3.0×10^6 Thiamine Nitrate 0.3 mg Riboflavin 0.2 mg Ascorbic Acid 5.0 mg Nicotinamide 0.1 mg Dibasic calcium phosphate 20.0 mg Dried yeast 50.0 mg

seems likely that spores are designed to survive transit across the gastric barrier of animals that ingest them. This view originates from studies that show that a percentage (>10%) of an inoculum of *B. subtilis* spores can germinate in the small intestine, grow and proliferate and then re-sporulate (Hoa et al., 2001; Tam et al., 2006). Peristalsis ensures that spores are shed in faeces resulting in their accumulation in the soil. An intestinal habitat of spore formers helps explain why spores can be found in the gut of insects, animals and humans (Barbosa et al., 2005; Fakhry et al., 2008; Hong et al., 2009a). Recent work has shown that *Bacilli* can readily be obtained from the human GI-tract using analysis of both biopsies and faeces (Fakhry et al., 2008; Hong et al., 2009a). In the latter, *Bacillus* spores can be found at levels of approximately 10^4 spores/g of faeces which is several logs higher than can reasonably be predicted from food intake alone (Hong et al., 2009b).

Numerous studies have shown that germinating spores can elicit potent immune responses in the GI-tract of mouse models and this immune stimulation may be the underlying reason why spores exert a probiotic effect (Hong et al., submitted for publication). One of the most informative, yet least recognised studies was one examining the effect of orally administered bacteria on the development of the gut-associated lymphoid tissue (GALT) in infant rabbits (Rhee et al., 2004). In these studies *B. subtilis* was shown to be of greater importance than other commensal bacteria in GALT development. Of course, other properties such as the secretion of antimicrobials such as Coagulin, Amicoumacin and Subtilisin may also further provide a probiotic effect by suppressing growth of competing microbes as well as enteric pathogens. Studies showing efficacy are less easy to distil yet a few convincing examples are as follows. In a poultry model *B. subtilis* spores were shown to suppress infection with pathogenic *S. enterica* (La Ragione and Woodward, 2003), *C. perfringens* (La Ragione and Woodward, 2003) and *E. coli* (La Ragione et al., 2001). A mouse model has been used to show suppression of *Citrobacter rodentium* (a model for the traveller's diarrhoea pathogen, ETEC) by administration of *B. subtilis* spores (D'Arienzo et al., 2006).

4. Safety

Two spore formers, *Bacillus anthracis* and *B. cereus* are known as human pathogens. The former requires no elaboration while the use of *B. cereus* appears to be a cause for concern on a case-by-case basis. The safety of *Bacillus* species has been extensively reviewed elsewhere (de Boer and Diderichsen, 1991; Ishibashi and Yamazaki, 2001; Logan, 2004; Osipova et al., 1998; Sanders et al., 2003; SCAN, 2000a) and most incidences of illness associated with *Bacillus* appear to result for opportunistic infections or miss-diagnosis. Extensive animal studies including acute and sub-chronic toxicity testing as well as *in vitro* studies have now been performed on a number of species, including *B. subtilis* var. Natto (Hong et al., 2008), *Bacillus indicus* (Hong et al., 2008), *B. coagulans* (Endres et al., 2009) and *B. subtilis 2335* (Sorokulova et al., 2008) and *B. licheniformis 2336* (Sorokulova et al., 2008). All appear to show no indicators of adverse effects.

5. Approved products in Europe and the USA

Bacillus products that have been formally approved in the West are few. Numerous authors routinely cite *B. subtilis* as having GRAS (Generally Regarded as Safe) status but this is incorrect. Nattokinase,

the proteolytic enzyme that is purified from *B. subtilis* var. Natto does carry GRAS status as a microbially produced enzyme but not the bacterium. In 2008 *B. coagulans* strain GanedenBC^{30} was the first *Bacillus* strain to be given self-affirmed GRAS approval. In Europe, for approval, for use as a supplement a case must be made based on prior use. The application is first made by authorities in the host country and then assessed by an EU committee. To date *B. subtilis* has been approved for use as a supplement in Italy and the UK. *B. clausii* that is used in the medicinal OTC product Enterogermina® and *B. cereus* IP5832 (Bactisubtil®) are registered as medicines with specific claims regarding the prevention of childhood diarrhoea and, as a medicine, are not marketed under the probiotic label.

6. The Vietnamese market

In SE Asia, notably, Vietnam, where no concept of dietary supplements exists, *Bacillus* products are licensed with the Ministry of Health as medicinal supplements (Table 4) with claims ranging from prevention of rotavirus infection (infant diarrhoea) and food poisoning to immune stimulation. It is unclear whether their approval requires formal clinical trials but in any event these products are easily obtained and often used as the first line of defence against enteric infections both prophylactically but more often therapeutically. The use of *Bacillus* probiotics in Vietnam is more developed than in any other country and the reason for this is unclear. There is also intense interest in using heat-stable *Bacillus* spores in aquaculture and it is not uncommon for shrimp farms to use products produced for human use.

7. Recent innovations: functional foods

In recent work pigmented *Bacillus* species have been characterised and the pigment has been shown to be due one or more carotenoids (Duc et al., 2006; Khaneja et al., 2009). These carotenoids have been shown to carry anti-oxidant activity *in vitro* and thus could be of nutritional value (SM Cutting; unpublished data). Yellow, orange, red and pink *Bacillus* species can be easily obtained from soil, river and pond sediments as well as from the intestinal tracts of animals (Hong et al., 2009a; Yoon et al., 2001, 2005). This includes a red pigmented *Bacillus megaterium* (Mitchell et al., 1986) a pink pigment found in some isolates of *Bacillus firmus* (Pane et al., 1996) and red pigment found in *Bacillus atrophaeus* (Fritze and Pukall, 2001; Nakamura, 1989). A variable yellow-orange pigmentation has been found in a number of species including, *B. indicus* (Suresh et al., 2004), *Bacillus cibi* (Yoon et al., 2005), *Bacillus vedderi* (Agnew et al., 1995), *Bacillus jeogali* (Yoon et al., 2001), *Bacillus okuhidensis* (Li et al., 2002), *Bacillus clarkii* (Nielsen et al., 1995), *Bacillus pseudofirmus* (Nielsen et al., 1995) and *B. firmus* (Ruger and Koploy, 1980). The carotenoids are found in the vegetative cell as well as in the spore and they help protect spores from UV radiation (Khaneja et al., 2009). It is no surprise that *Bacillus* species found in aquatic environments and the animals that inhabit these environments are often rich in carotenoids. Carotenoids are of nutritional value and used as dietary supplements. When used as supplements the recommended daily allowance of carotenoids is often quite high (e.g., 800 mg/day for β-carotene). The reason for this is that carotenoids are rapidly degraded in the stomach which raises questions over their nutritional value. Spore carotenoids though appear to be gastric stable and studies currently in progress are designed to establish the uptake of spore carotenoids using *in vitro* and *in vivo* models (SM Cutting, unpublished data). It is apparent that carotenoid-rich spores could be used commercially as dietary supplements providing a source of carotenoids as well as conferring probiotic properties.

A further development with spore probiotics is that they can survive mild heat-treatments used to sterilise food. In principle, spores could be added to beverages and foods yet retain their probiotic properties. Indeed, such probiotic foods have already entered the market with “Activate Muffins” containing GanedenBC^{30} launched by Isabella’s Health Bakery in the USA in 2008.

8. Conclusions

The use of *Bacillus* species as probiotic dietary supplements is expanding rapidly with increasing number of studies demonstrating immune stimulation, antimicrobial activities and competitive exclusion. The single and most important advantage of these products is that they can be produced easily and the stability of the finished product can be assured, further they can be incorporated into everyday foods. Studies are showing that these bacteria are able to grow within the intestinal tract and possibly be considered temporary residents. This is important because it shows that these bacteria are not foreigners but rather may exert a unique symbiotic relationship with their host.

Acknowledgements

This article was based in part on a publication in Nutrafoods (2009 Vol. 8:7–14). Research in the laboratory of SMC and TCD is supported by an EU 7th FP grant, KBBE-2007-207948.

References

Agnew, M.D., Koval, S.F., Jarrell, K.F., 1995. Isolation and characterisation of novel alkaliphiles from bauxite-processing waste and description of *Bacillus vedderi* sp. nov., a new obligate alkaliphile. Syst. Appl. Microbiol. 18, 221–230.

Araya, M., Morelli, L., Reid, G., Sanders, M.E., Stanton, C., 2002. Joint FAO/WHO Working Group Report on Guidelines for the Evaluation of Probiotics in Food London, Ontario. ftp://ftp.fao.org/es/esn/food/wgreport2.pdf.

Barbosa, T.M., Serra, C.R., La Ragione, R.M., Woodward, M.J., Henriques, A.O., 2005. Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Appl. Environ. Microbiol. 71, 968–978.

Bilev, A.E., 2002. Comparative evaluation of probiotic activity in respect to in vitro pneumotropic bacteria and pharmacodynamics of biosporin-strain producers in patients with chronic obstructive pulmonary diseases. Voen. Med. Zh. 323, 54–57.

Bozdogan, B., Galopin, S., Leclereq, R., 2004. Characterization of a new erm-related macrolide resistance gene present in probiotic strains of *Bacillus clausii*. Appl. Environ. Microbiol. 70, 280–284.

Ciffo, F., 1984. Determination of the spectrum of antibiotic resistance of the *Bacillus subtilis* strains of Enterogermina. Chemioterapia 3, 45–52.

Ciprandi, G., Tosca, M.A., Milanese, M., Caligo, G., Ricca, V., 2004. Cytokines evaluation in nasal lavage of allergic children after *Bacillus clausii* administration: a pilot study. Pediatr. Allergy Immunol. 15, 148–151.

Ciprandi, G., Vizzaccaro, A., Cirillo, I., Tosca, M.A., 2005a. *Bacillus clausii* effects in children with allergic rhinitis. Allergy 60, 702–703.

Ciprandi, G., Vizzaccaro, A., Cirillo, I., Tosca, M.A., 2005b. *Bacillus clausii* exerts immuno-modulatory activity in allergic subjects: a pilot study. Eur. Ann. Allergy Clin. Immunol. 37, 129–134.

Coppi, F., Ruoppolo, M., Mandressi, A., Bellorofonte, C., Gonnella, G., Trinchieri, A., 1985. Results of treatment with *Bacillus subtilis* spores (Enterogermina) after antibiotic therapy in 95 patients with infection calculosis. Chemioterapia 4, 467–470.

D’Arienzo, R., Maurano, F., Mazzarella, G., Luongo, D., Stefanile, R., Ricca, E., Rossi, M., 2006. *Bacillus subtilis* spores reduce susceptibility to *Citrobacter rodentium*-mediated enteropathy in a mouse model. Res. Microbiol. 157, 891–897.

de Boer, A.S., Diderichsen, B., 1991. On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review. Appl. Microbiol. Biotechnol. 36, 1–4.

Drago, L., De Vecchi, E., 2009. Should *Lactobacillus sporogenes* and *Bacillus coagulans* have a future? J. Chemother. 21, 371–377.

Duc, L.H., Fraser, P., Cutting, S.M., 2006. Carotenoids present in halotolerant *Bacillus* spore formers. FEMS Microbiol. Lett. 255, 215–224.

Endres, J.R., Clewell, A., Jade, K.A., Farber, T., Hauswirth, J., Schauss, A.G., 2009. Safety assessment of a proprietary preparation of a novel probiotic, *Bacillus coagulans*, as a food ingredient. Food Chem. Toxicol..

Errington, J., 2003. Regulation of endospore formation in *Bacillus subtilis*. Nat. Rev. Microbiol. 1, 117–126.

Fakhry, S., Sorrentini, I., Ricca, E., De Felice, M., Baccigalupi, L., 2008. Characterization of spore forming *Bacilli* isolated from the human gastrointestinal tract. J. Appl. Microbiol. 105, 2178–2186.

Fiorini, G., Cimminiello, C., Chianese, R., Visconti, G.P., Cova, D., Uberti, T., Gibelli, A., 1985. *Bacillus subtilis* selectively stimulates the synthesis of membrane bound and secreted IgA. Chemioterapia 4, 310–312.

Fritze, D., Pukall, R., 2001. Reclassification of bioindicator strains *Bacillus subtilis* DSM 675 and *Bacillus subtilis* DSM 2277 as *Bacillus atrophaeus*. Int. J. Syst. Evol. Microbiol. 51, 35–37.

Green, D.H., Wakeley, P.R., Page, A., Barnes, A., Baccigalupi, L., Ricca, E., Cutting, S.M., 1999. Characterization of two *Bacillus* probiotics. Appl. Environ. Microbiol. 65, 4288–4291.

Henriques, A.O., Moran Jr., C.P., 2007. Structure, assembly, and function of the spore surface layers. Annu. Rev. Microbiol. 61, 555–588.

Hoa, N.T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.H., Ammendola, S., Ricca, E., Cutting, S.M., 2000. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl. Environ. Microbiol. 66, 5241–5247.

Hoa, T.T., Duc, L.H., Isticato, R., Baccigalupi, L., Ricca, E., Van, P.H., Cutting, S.M., 2001. Fate and dissemination of *Bacillus subtilis* spores in a murine model. Appl. Environ. Microbiol. 67, 3819–3823.

Hong, H.A., Duc le, H., Cutting, S.M., 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. 29, 813–835.

Hong, H.A., Huang, J.-M., Khaneja, R., Hiep, L.V., Urdaci, M.C., Cutting, S.M., 2008. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. J. Appl. Microbiol. 105, 510–520.

Hong, H.A., Khaneja, R., Tam, N.M., Cazzato, A., Tan, S., Urdaci, M., Brisson, A., Gasbarrini, A., Barnes, I., Cutting, S.M., 2009a. *Bacillus subtilis* isolated from the human gastrointestinal tract. Res. Microbiol. 160, 134–143.

Hong, H.A., To, E., Fakhry, S., Baccigalupi, L., Ricca, E., Cutting, S.M., 2009b. Defining the natural habitat of *Bacillus* spore-formers. Res. Microbiol..

Hong, A.H., Duc, H.L., Cutting, S.M. Immunogenicity and intracellular fate of *Bacillus subtilis* spores. Microbiology, submitted for publication.

Hosoi, T., Kiuchi, K., 2004. Production and probiotic effects of Natto. In: Ricca, E., Henriques, A.O., Cutting, S.M. (Eds.), Horizon Bioscience, pp. 143–154. Wymondham, UK.

Hyronimus, B., Le Marrec, C., Urdaci, M.C., 1998. Coagulin, a bacteriocin-like inhibitory substance produced by *Bacillus coagulans* I4. J. Appl. Microbiol. 85, 42–50.

Ishibashi, N., Yamazaki, S., 2001. Probiotics and safety. Am. J. Clin. Nutr. 73, 465S–470S.

Khaneja, R., Perez-Fons, L., Fakhry, S., Baccigalupi, L., Steiger, S., To, E., Sandmann, G., Dong, T.C., Ricca, E., Fraser, P.D., Cutting, S.M., 2009. Carotenoids found in *Bacillus*. J. Appl. Microbiol..

La Ragione, R.M., Casula, G., Cutting, S.M., Woodward, M., 2001. *Bacillus subtilis* spores competitively exclude *Escherichia coli* 070:K80 in poultry. Vet. Microbiol. 79, 133–142.

La Ragione, R.M., Woodward, M.J., 2003. Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype *Enteritidis* and *Clostridium perfringens* in young chickens. Vet. Microbiol. 94, 245–256.

Li, Z., Kawamura, Y., Shida, O., Yamagata, S., Deguchi, T., Ezaki, T., 2002. *Bacillus okuhidensis* sp. nov., isolated from the Okuhida spa area of Japan. Int. J. Syst. Evol. Microbiol. 52, 1205–1209.

Logan, N.A., 2004. Safety of aerobic endospore-forming bacteria. In: Ricca, E., Henriques, A.O., Cutting, S.M. (Eds.), Bacterial Spore Formers: Probiotics and Emerging Applications. Horizon Bioscience, pp. 93–106. Norfolk, UK.

Mandel, D.R., Eichas, K., Holmes, J., 2010. *Bacillus coagulans*: a viable adjunct therapy for relieving symptoms of rheumatoid arthritis according to a randomized, controlled trial. BMC Complement. Altern. Med. 10, 1.

Marseglia, G.L., Tosca, M., Cirillo, I., Licari, A., Leone, M., Marseglia, A., Castellazzi, A.M., Ciprandi, G., 2007. Efficacy of *Bacillus clausii* spores in the prevention of recurrent respiratory infections in children: a pilot study. Ther. Clin. Risk Manag. 3, 13–17.

Mazza, G., 1983. Genetic studies on the transfer of antibiotic resistance genes in *Bacillus subtilis* strains. Chemioterapia 2, 64–72.

Mazza, P., 1994. The use of *Bacillus subtilis* as an antidiarrhoeal microorganism. Boll. Chim. Farm. 133, 3–18.

Mazza, P., Zani, F., Martelli, P., 1992. Studies on the antibiotic resistance of *Bacillus subtilis* strains used in oral bacteriotherapy. Boll. Chim. Farm. 131, 401–408.

Mitchell, C., Iyer, S., Skomurski, J.F., Vary, J.C., 1986. Red pigment in *Bacillus megaterium* spores. Appl. Environ. Microbiol. 52, 64–67.

Moir, A., 2006. How do spores germinate? J. Appl. Microbiol. 101, 526–530.

Nakamura, L.K., 1989. Taxonomic relationship of black-pigmented *Bacillus subtilis* strains and a proposal for *Bacillus atrophaeus* sp. nov. Int. J. Syst. Bacteriol. 39, 295–300.

Nicholson, W.J., Munakata, N., Horneck, G., Melosh, H.J., Setlow, P., 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64, 548–572.

Nielsen, P., Fritze, D., Priest, F.G., 1995. Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. Microbiology 141, 1745–1761.

Nista, E.C., Candelli, M., Cremonini, F., Cazzato, I.A., Zocco, M.A., Franceschi, F., Cammarota, G., Gasbarrini, G., Gasbarrini, A., 2004. *Bacillus clausii* therapy to reduce side-effects of anti-*Helicobacter pylori* treatment: randomized, double-blind, placebo controlled trial. Aliment. Pharmacol. Ther. 20, 1181–1188.

Osipova, I.G., Sorokulova, I.B., Tereshkina, N.V., Grigor'eva, L.V., 1998. Safety of bacteria of the genus *Bacillus*, forming the base of some probiotics. Zh Mikrobiol Epidemiol. Immunobiol 6, 68–70.

Osipova, I.G., Makhailova, N.A., Sorokulova, I.B., Vasil'eva, E.A., Gaiderov, A.A., 2003. Spore probiotics. Zh. Mikrobiol. Epidemiol. Immunobiol., 113–119.

Osipova, I.G., Sorokulova, I.B., Vasil'eva, E.A., Budanova, E.V., 2005. Pre-clinical trials of new spore probiotics. Vestn. Ross. Akad. Med. Nauk, 36–40.

Pane, L., Radin, L., Franconi, G., Carli, A., 1996. The carotenoid pigments of a marine *Bacillus firmus* strain. Boll. Soc. Ital Biol. Sper. 72, 303–308.

Pinchuk, I.V., Bressollier, P., Verneuil, B., Fenet, B., Sorokulova, I.B., Megraud, F., Urdaci, M.C., 2001. *In vitro* anti-*Helicobacter pylori* activity of the probiotic strain *Bacillus subtilis* 3 is due to secretion of antibiotics. Antimicrob. Agents Chemother. 45, 3156–3161.

Rhee, K.J., Sethupathi, P., Driks, A., Lanning, D.K., Knight, K.L., 2004. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. J. Immunol. 172, 1118–1124.

Ruger, H.-J., Koploy, J.A.C., 1980. DNA base composition of halophilic and non-halophilkic *Bacillus firmus* strains of marine origin. Microb. Ecol. 6, 141–146.

Sanders, M.E., Morelli, L., Tompkins, T.A., 2003. Sporeformers as human probiotics: *Bacillus*, *Sporolactobacillus*, and *Brevibacillus*. Compr. Rev. Food Sci. Food Saf. 2, 101–110.

SCAN, 2000a. Opinion of the Scientific Committee on Animal Nutrition on the Safety of the Use of *Bacillus* Species in Animal Nutrition. European Commission, Health and Consumer Protection Directorate-General. (SCAN) Scientific Committee on Animal Nutrition.

SCAN, 2000b. Report of the Scientific Committee on Animal Nutrition on Product BioPlus 2B® for Use as Feed Additive. European Commission, Health and Consumer Protection Directorate-General. (SCAN) Scientific Committee on Animal Nutrition.

SCAN, 2001a. Assessment by the Scientific Committee on Animal Nutrition of the Safety of Product Paciflor® for Use as Feed Additive. European Commission, Health and Consumer Protection Directorate-General. (SCAN) Scientific Committee on Animal Nutrition.

SCAN, 2001b. Report of the Scientific Committee on Animal Nutrition on Product Toyocerin® for Use as Feed Additive. European Commission, Health and Consumer Protection Directorate-General. (SCAN) Scientific Committee on Animal Nutrition.

Senesi, S., Celandroni, F., Tavanti, A., Ghelardi, E., 2001. Molecular characterization and identification of *Bacillus clausii* strains marketed for use in oral bacteriotherapy. Appl. Environ. Microbiol. 67, 834–839.

Sorokulova, I.B., 1997. A comparative study of the biological properties of Biosporin and other commercial *Bacillus*-based preparations. Mikrobiol. Zh. 59, 43–49.

Sorokulova, I.B., Beliavskaia, V.A., Masycheva, V.A., Smirnov, V.V., 1997. Recombinant probiotics: problems and prospects of their use for medicine and veterinary practice. Vestn. Ross. Akad. Med. Nauk, 46–49.

Sorokulova, I.B., Pinchuk, I.V., Denayrolles, M., Osipova, I.G., Huang, J.M., Cutting, S.M., Urdaci, M.C., 2008. The safety of two *Bacillus* probiotic strains for human use. Dig. Dis. Sci. 53, 954–963.

Spinosa, M.R., Braccini, T., Ricca, E., De Felice, M., Morelli, L., Pozzi, G., Oggioni, M.R., 2000. On the fate of ingested *Bacillus* spores. Res. Microbiol. 151, 361–368.

Stenfors Arnesen, L.P., Fagerlund, A., Granum, P.E., 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol. Rev. 32, 579–606.

Sumi, H., Hamada, H., Tsushima, H., Mihara, H., Muraki, H., 1987. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. Experientia 43, 1110–1111.

Sumi, H., Yatagai, C., Wada, H., Yoshida, E., Maruyama, M., 1995. Effect of *Bacillus natto*-fermented product (BIOZYME) on blood alcohol, aldehyde concentrations after whisky drinking in human volunteers, and acute toxicity of acetaldehyde in mice. Arukoru Kenkyuto Yakubutsu Ison 30, 69–79.

Suresh, K., Prabagaran, S.R., Sengupta, S., Shivaji, S., 2004. *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India. Int. J. Syst. Evol. Microbiol. 54, 1369–1375.

Tam, N.K., Uyen, N.Q., Hong, H.A., Duc le, H., Hoa, T.T., Serra, C.R., Henriques, A.O., Cutting, S.M., 2006. The intestinal life cycle of *Bacillus subtilis* and close relatives. J. Bacteriol. 188, 2692–2700.

Tuohy, K.M., Pinart-Gilberga, M., Jones, M., Hoyles, L., McCartney, A.L., Gibson, G.R., 2007. Survivability of a probiotic *Lactobacillus casei* in the gastrointestinal tract of healthy human volunteers and its impact on the faecal microflora. J. Appl. Microbiol. 102, 1026–1032.

Yoon, J.H., Kang, S.S., Lee, K.C., Kho, Y.H., Choi, S.H., Kang, K.H., Park, Y.H., 2001. *Bacillus jeotgali* sp. nov., isolated from jeotgal, Korean traditional fermented seafood. Int. J. Syst. Evol. Microbiol. 51, 1087–1092.

Yoon, J.H., Lee, C.H., Oh, T.K., 2005. *Bacillus cibi* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. Int. J. Syst. Evol. Microbiol. 55, 733–736.