Review

Bacillus probiotics

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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.

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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 Clostridium 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.
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 × 10⁹) 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 (Cifó, 1984; Mazza, 1994). Interestingly, these B. clausii strains also carry resistance to a number of other antibiotics including erythromycin, cephalosporins and ciprofloxacin, 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**

<table>
<thead>
<tr>
<th>Bacillus probiotics for human use.</th>
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<tbody>
<tr>
<td><strong>Product</strong></td>
</tr>
<tr>
<td>Bactisubtil®</td>
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<tr>
<td>Bio-Kult®</td>
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<tr>
<td>Biosporin®</td>
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<td></td>
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<td></td>
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<tr>
<td>Biovicerin®</td>
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<tr>
<td>Bispan®</td>
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<tr>
<td>Donovar</td>
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<tr>
<td>Enterogermina®</td>
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<tr>
<td>Flora-Balance</td>
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<tr>
<td>Sustenex®</td>
</tr>
<tr>
<td>Lactipan Plus</td>
</tr>
<tr>
<td>Lactospore</td>
</tr>
<tr>
<td>Medilac-Vita</td>
</tr>
<tr>
<td>Nature’s First Food</td>
</tr>
<tr>
<td>Neolactoflorense</td>
</tr>
<tr>
<td>Primal Defense™</td>
</tr>
</tbody>
</table>

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

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.
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 GanedenBC30 has been granted self-affirmed GRAS status by the FDA in the US. Marketed by Ganeden, as GanedenBC30 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 stabilise 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 2

<table>
<thead>
<tr>
<th>Brand</th>
<th>Animal</th>
<th>Manufacturer</th>
<th>Comments</th>
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<tbody>
<tr>
<td>AliCare</td>
<td>Swine</td>
<td>Alpharma Inc, Melbourne, Australia</td>
<td>B. licheniformis (NCTC 13123) at 10^8-10^10 spores kg⁻¹, This is a non-bacitracin producing strain. Not licensed in the EU. Listed as containing spores of B. licheniformis (1.6 × 10^8 CFU g⁻¹) and B. subtilis (1.6 × 10^6 CFU g⁻¹).</td>
</tr>
<tr>
<td>BioPlus 2B</td>
<td>Piglets, turkeys for fattening</td>
<td>Danish bacteria (DSM 5749) and B. subtilis (DSM 5750) at 1.6 × 10^8 CFU g⁻¹ of each bacterium. EU approved. Listed as carrying spores of B. licheniformis (1.6 × 10^8 CFU g⁻¹) and B. subtilis (1.6 × 10^6 CFU g⁻¹).</td>
<td></td>
</tr>
<tr>
<td>Lactopure</td>
<td>Swine</td>
<td>Christian Hansen Hydrocolloid, Denmark <a href="http://www.chbioystems.com">http://www.chbioystems.com</a></td>
<td></td>
</tr>
<tr>
<td>Neurof 10</td>
<td>Poultry, calves and swine</td>
<td>Sanofi Sante Nutrition Animale, France</td>
<td></td>
</tr>
<tr>
<td>Toyocerin</td>
<td>Calves, poultry, rabbits and swine</td>
<td>Asahi Vet S.A., Tokyo (Head Off.), Japan <a href="http://www.asahi-kasei.co.jp">http://www.asahi-kasei.co.jp</a></td>
<td></td>
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Table 3

<table>
<thead>
<tr>
<th>Brand</th>
<th>Manufacturer</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaoZyme-Aqua</td>
<td>Sino-Aqua Corp., Kaohsiung, Taiwan</td>
<td>B. subtilis strains Wu-S and Wu-T at 10^8 CFU g⁻¹, product also contains Lactobacillus and Saccharomyces spp.</td>
</tr>
<tr>
<td>Biostart</td>
<td><a href="http://www.sino-aqua.com">www.sino-aqua.com</a> Advanced Microbial Solutions, Shalkeopee, MN, USA</td>
<td></td>
</tr>
<tr>
<td>Liquiface</td>
<td>Cargill, Animal Nutrition Division</td>
<td></td>
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<tr>
<td>Promarine</td>
<td><a href="http://www.sino-aqua.com">www.sino-aqua.com</a> Sino-Aqua company Kaohsiung, Taiwan</td>
<td></td>
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<tr>
<td>Sanocare Sanolife Sanoguard</td>
<td>INVE Technologies nv Dendermonde, Belgium <a href="http://www.inve.com">www.inve.com</a></td>
<td></td>
</tr>
</tbody>
</table>

References:

Marseglia et al., 2007: 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.

Fiorini et al., 1985: Product originally labelled as carrying spores of B. subtilis but subsequent studies have identified the species as B. clausii.

Green et al., 1999: B. subtilis clausii is not a Bacillus species (www.bacterio.cict.fr).

Senesi et al., 2001: Recently one strain, labelled as GanedenBC30 has been granted self-affirmed GRAS status by the FDA in the US. Marketed by Ganeden, as GanedenBC30 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 stabilise 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).

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Table 2: Bacillus probiotics for veterinary use.*

* Authorised for unlimited use by the EU.

Table 3: Bacillus probiotics for aquaculture.*

* 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.
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/C210. BioPlus/C210 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 have 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 *H. pylori* (Pinchuk et al., 2001). In the case of BioPlus/C210 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, Pacificlor, 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 Pacificlor®.

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
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 proliferation and then re-sporulate (Hoa et al., 2001; Tam et al., 2006).

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 Bacillus spores mixed with tapioca. Each gram of granules contains:

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 testing as well as a cause for concern on a case-by-case basis.

### 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 GaneDenBC30 was the first Bacillus strain to be given self-confirmed 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 (Bactisubtilis®) 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 *vivo* 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, 1991; 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 vederdi* (Agnew et al., 1995), *Bacillus jeogali* (Yoon et al., 2001), *Bacillus okudakensis* (Li et al., 2002), *Bacillus clarkii* (Nielsen et al., 1995), *Bacillus pseudo- firmus* (Nielsen et al., 1995) and *B. firmus* (Ruger and Klopoy, 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 *vivo* 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 GaneDenBC30 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

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References


