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). 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.

B. clausii

B. clausii spores are used in the product Enterogermina® which is registered as an OTC medicinal supplement (**Fig. 3**). Unlike most probiotic formulations that are supplied in tablet or capsule form the Enterogermina product carries, spores (2×10^9) suspended in 5ml of water and 2-3 vials are taken each day with the aim of preventing infantile diarrhoea. 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).

Figure 3: Enterogermina®.

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; CIPRANDI et al., 2005a; CIPRANDI et al., 2005b). After administration of the probiotic nasal symptoms and eosinophil counts in allergic 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 (MARSEGLIA et al., 2007) who have examined the duration and rate of respiratory infections in 40 children (mean age 4.3 ± 1.5 yr). 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.

B. coagulans

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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).

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) (Fig. 4).

Figure 4: Natto.

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; SUMI et al., 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 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 bacetrium. 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; OSIPOVA et al., 2005; SOROKULOVA, 1997; SOROKULOVA et al., 1997). Interestingly, *B. subtilis 2335* has been shown to produce the antibiotic Amicoumacin with *in vitro* activity against *Heliobacter 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.

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 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., 2002). 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 commnsal 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 *Salmonella enterica* (LA RAGIONE and WOODWARD, 2003), *Clostridium 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).

Safety

Two spore formers, *B. 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), *B. 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.

Figure 5: Biosubtyl and Biosubtyl DL.

*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.

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; YOON et al., 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), *B. cibi* (YOON et al., 2005), *B. vedderi* (AGNEW et al., 1995), *B. jeogali* (YOON et al., 2001), *B. okuhidensis* (LI et al., 2002), *B. clarkii* (NIELSEN et al., 1995), *B. 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.

Conclusions

The use of *Bacillus* species as probiotic dietary/food 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.