

The use of bacterial spore formers as probiotics

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Abstract

The field of probiosis has emerged as a new science with applications in farming and aquaculture as alternatives to antibiotics as well as prophylactics in humans. Probiotics are being developed commercially for both human use, primarily as novel foods or dietary supplements, and in animal feeds for the prevention of gastrointestinal infections, with extensive use in the poultry and aquaculture industries. The impending ban of antibiotics in animal feed, the current concern over the spread of antibiotic resistance genes, the failure to identify new antibiotics and the inherent problems with developing new vaccines make a compelling case for developing alternative prophylactics. Among the large number of probiotic products in use today are bacterial spore formers, mostly of the genus *Bacillus*. Used primarily in their spore form, these products have been shown to prevent gastrointestinal disorders and the diversity of species used and their applications are astonishing. Understanding the nature of this probiotic effect is complicated, not only because of the complexities of understanding the microbial interactions that occur within the gastrointestinal tract (GIT), but also because *Bacillus* species are considered allochthonous microorganisms. This review summarizes the commercial applications of *Bacillus* probiotics. A case will be made that many *Bacillus* species should not be considered allochthonous microorganisms but, instead, ones that have a bimodal life cycle of growth and sporulation in the environment as well as within the GIT. Specific mechanisms for how *Bacillus* species can inhibit gastrointestinal infections will be covered, including immunomodulation and the synthesis of antimicrobials. Finally, the safety and licensing issues that affect the use of *Bacillus* species for commercial development will be summarized, together with evidence showing the growing need to evaluate the safety of individual *Bacillus* strains as well as species on a case by case basis.

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Keywords: *Bacillus*; Probiotic; Spores; Immunomodulation

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1. Introduction

Probiotics, although not a new concept, has only recently begun to receive an increasing level of scientific interest. Probiotics are generally defined as 'live microbial feed supplements that can benefit the host by improving its intestinal balance' [1]. Probiotics fall under two broad classifications, those for animal use and those for human use. Probiotics used in animal feed are considered as alternatives to antibiotics (and therefore used as growth promoters). In 2000 Denmark banned the use of antibiotics as growth promoters in its pig industry and 2006 is the date proposed for a complete ban of antibiotics in animal feed within Europe [2]. A viable alternative to antibiotics would therefore be an important venture and for this reason the development of new probiotic products that could be licensed for animal use is receiving considerable interest. However, the transfer of antibiotic resistance traits between bacterial species is a cause for concern where large quantities of bacteria would be given to animals. In Europe it is estimated that to licence a new probiotic product for use in animal feed requires upwards of 1.4 million Euros [3]. Probiotics for human use, on the other hand, are subject to minimal restrictions (at least as novel foods or as dietary supplements) and come in many different forms. In supermarkets they are often sold as dairy-type products containing 'live bacteria' and in health food shops as

capsules or tablets composed of lyophilized preparations of bacteria which promote 'a healthy gut', etc. Finally, on the internet some products are being sold as quasi-medicinal products which can be used for oral bacteriotherapy of gastrointestinal disorders (normally diarrhoea).

Currently, there is no universal 'class' of probiotic bacterium although the most common types available are lactic acid bacteria (e.g., *Lactobacillus* spp.). These bacteria are found normally in the gastrointestinal tract (GIT) of humans and animals and there is the vague notion that the use of indigenous or commensal microorganisms is somehow restoring the natural microflora to the gut. A second class comprises those that are not normally found in the GIT. For example, *Saccharomyces boulardii* has been shown to be effective in preventing the recurrence of *Clostridium difficile*-induced pseudomembranous colitis [4] as well as the antagonistic action of *Escherichia coli* [5]. *S. boulardii* products are currently being marketed for human use. Within this group of allochthonous probiotic microbes are the spore-forming bacteria, normally members of the genus *Bacillus*. Here, the product is used in the spore form and thus can be stored indefinitely 'on the shelf'. The use of spore-based products raises a number of questions though. Since the bacterial species being used are not considered resident members of the gastrointestinal microflora how do they exert a beneficial effect? Because the natural life cycle of

spore formers involves germination of the spore, proliferation and then re-sporulation when nutrients are exhausted, the logical question is whether it is the germinated spore (that is the vegetative cell) that produces the probiotic effect or is it the spore itself? If the former model is correct then it would suggest that probiotics show a unified mode of action involving the action of a *live bacterium* within the GIT. This review, based on published studies, will present the case that spore-forming bacteria can survive and, indeed, proliferate within the GIT of animals. Although it is unlikely that they are true commensals, a case will be made that many spore formers exhibit a unique dual life cycle of growth and survival in both the environment and within the gut of animals and it is this bimodal life cycle that could provide the basis of their probiotic effect.

This review will focus exclusively on the use of spore-forming bacteria as probiotics for human and animal use. For conciseness, with a few exceptions, this review will only report on studies relating to species used in existing commercial formulations and will cover their use in humans and animals, as well as in aquaculture. Finally, it should be mentioned that this review expands on two excellent reviews in this field [6,7].

2. Commercial products

A list of *Bacillus* probiotic products (probably not complete) is shown in Table 1 and these are discussed where appropriate in more detail throughout this review.

2.1. Human products

Products fall into two major groups, those for prophylactic use and those sold as health food supplements or novel foods. *Bona fide Bacillus* species being used include, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus clausii* and *Bacillus coagulans*. Other spore-formers being used are *Paenibacillus polymyxa* and *Brevibacillus laterosporus*, both being former *Bacillus* species and now belonging to the *Bacillus sensu lato* group.

2.1.1. Prophylactics

These are marketed for prophylaxis of gastrointestinal disorders particularly child-hood diarrhoea (mainly rotavirus infections) or as an adjunct to antibiotic use. These products are available over the counter (OTC) and, very often, they have been recommended by a physician. Their use then, depends very much upon the national or local culture. For example, in the UK no probiotics for human use as prophylactics for gastrointestinal disorders are available, yet in Europe they are quite common with Italy being a major user. One of

the oldest products on the market and available in Italy since the 1950s is Enterogermina[®] which carries a mixture of four strains of antibiotic-resistant *B. clausii*, an alkaliphilic species able to tolerate high pH [7–14].

Another well-known product is Bactisubtil[®] which carries one strain of *B. cereus* termed IP5832 [7,8,12,14–17]. The same strain (labeled as CIP 5832) has been used in the animal feed product Paciflor[®] C10 that has recently been withdrawn due to the ability of this strain to produce two diarrhoea enterotoxins, Hbl and Nhe [18,19]. It remains unclear whether this product will remain in use for humans.

Biosporin[®] carries spores of two *Bacillus* species, *B. subtilis* and *B. licheniformis*. This product is manufactured in a number of former Eastern bloc countries and appears to have been well characterized (see Table 1; [16,17,20–25]). The *B. subtilis* component of Biosporin[®] (*B. subtilis* strain 3 or 2335) is known to produce an isocourmarin antibiotic, aminocoumarin A, active against *Helicobacter pylori* [26]. The *B. subtilis* strain from Biosporin[®] has been genetically modified to express interferon and a new product, Subalin, carrying this recombinant is licensed in Russia (currently for veterinary use) with claims of both anti-viral and anti-tumour activity [17,27–29].

In SE Asia there is a history of extensive antibiotic usage and it is common practice in developing countries within this region to use probiotics as an adjunct. Consequently, there are now a large number of products being produced, all of which carry poorly defined species; e.g., Biosubtyl ‘Nha Trang’ (*B. pumilus*) [12,30], Biosubtyl ‘Da Lat’ (*B. cereus*) [12,19], Subtyl (*B. cereus*) [12,19], and Bibactyl (*B. subtilis*), but with most carrying substantial resistance to antibiotics! South Korea produces one product termed ‘Biscan’ that carries spores of *B. polyfermenticus* SCD (an invalid species name) [31]. China and India are also producing different probiotic products (see Table 1) and the origin and status of these products appears to be poorly defined [6].

2.1.2. Health foods and dietary supplements

A large number of *Bacillus* products are used as ‘novel foods’ or as dietary supplements with various claims of ‘enhancing’ the well-being of the user, restoring the natural microflora to the gut, etc. Many of these products are sold over the internet and many carry poorly defined or invalid species (e.g., *Bacillus laterosporus*, *Lactobacillus sporogenes*). Some products carry mixtures of *Bacillus* species (e.g. Nature’s First Food listing 42 species including 4 spore-forming species) [6].

It is worthwhile mentioning here the Japanese product Natto. Natto is a food made by fermenting cooked soybeans with *Bacillus subtilis* (*natto*) or *B. subtilis* var. *natto*. Natto has been shown to have probiotic properties and the *B. subtilis* var. *natto* component is thought to stimulate the immune system, produce vita-

Table 1
Commercial probiotic products containing *Bacillus* spores^a

Product	Target	Manufacturer	Comments/References
AlCare™	Swine	Alpharma Inc., Melbourne, Australia www.alpharma.com.au/alcare.htm	<i>B. licheniformis</i> (NCTC 13123) at 10 ⁹ –10 ¹⁰ spores kg ⁻¹ . This is a non-bacitracin producing strain. Not licensed in the EU [177].
Bactisubtil®	Human	Originally produced by Marion Merrell Dow Laboratories (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 <i>Bacillus cereus</i> strain IP 5832 ^b (ATCC 14893) [n.b., originally deposited as <i>B. subtilis</i> , 6,12,15,18].
BaoZyme-Aqua	Aquaculture-shrimps	Sino-Aqua Corp., Kaohsiung, Taiwan www.sino-aqua.com	<i>B. subtilis</i> strains Wu-S and Wu-T at 10 ⁸ CFU g ⁻¹ , product also contains <i>Lactobacillus</i> and <i>Saccharomyces</i> spp.
Bibactyl	Human	Tendiphar Corporation, Ho Chi Minh City, Vietnam	Sachet (1g) carrying 10 ⁷ –10 ⁸ spores of <i>B. subtilis</i> .
Bidisubtilis	Human	Bidiphar. Binh Dinh Pharmaceutical and Medical Equipment Company, 498 Nguyen Thai Hoc, Qui Nhon, Vietnam	Labelled sachets carrying 1 × 10 ⁶ spores of <i>B. subtilis</i> .
BioGrow®	Poultry, calves and swine	Provita Eurotech Ltd., Omagh, Northern Ireland, UK. http://www.provita.co.uk	Listed as containing spores of <i>B. licheniformis</i> (1.6 × 10 ⁹ CFU g ⁻¹) and <i>B. subtilis</i> (1.6 × 10 ⁹ CFU g ⁻¹).
BioPlus 2B®	Piglets ^b , Chickens, turkeys for fattening ^c	Christian Hansen Hoersholm, Denmark http://www.chbiosystems.com	Mixture (1/1) of <i>B. licheniformis</i> (DSM 5749) and <i>B. subtilis</i> (DSM 5750) at 1.6 × 10 ⁹ CFU g ⁻¹ of each bacterium. EU approved ^c [42].
Biosporin®	Human	(1) Biofarm, Dniepropetrovsk, Ukraine (2) Garars, Russia.	Biosporin® is a mixture of two strains of living antagonistic bacteria <i>B. subtilis</i> 2335 (sometimes referred to as <i>B. subtilis</i> 3) and <i>B. licheniformis</i> 2336 (ratio is 3:1). Originally isolated from animal fodder [15–17,20–26,182]. There are a number of versions of this product produced in different countries including a recombinant form, Subalin [27–29,183].
Biostart®	Aquaculture	Microbial Solutions, Johannesburg, South Africa and Advanced Microbial Systems, Shakopee, MN, USA	Mixture of: <i>B. megaterium</i> , <i>B. licheniformis</i> , <i>Paenibacillus polymyxa</i> and two strains of <i>B. subtilis</i> [45].
Biosubtyl	Human	Biophar Company, Da lat, Vietnam	Sachet (1 g) carrying 10 ⁶ –10 ⁷ of <i>B. cereus</i> spores mixed with tapioca. Product labelled as <i>B. subtilis</i> . The strain is closely related by 16S rRNA analysis to IP 5832 used in Bactisubtil® [12,19].
Biosubtyl DL	Human	IVAC, 18 Le Hong Phong, Da Lat, Vietnam.	Sachets (1g) carrying 10 ⁷ –10 ⁸ CFU of <i>B. subtilis</i> and <i>Lactobacillus acidophilus</i> .
Biosubtyl	Human	Biophar Company, Nha Trang, Vietnam	Sachet (1 g) carrying 10 ⁶ –10 ⁷ of <i>B. pumilus</i> spores mixed with tapioca. Product labelled as <i>B. subtilis</i> [11,12,19].
Biovicerin®	Human	Geyer Medicamentos S. A. Porto Alegre, RS, Brazil http://www.geyarmed.com	<i>B. cereus</i> strain GM Suspension of 10 ⁶ spores ml ⁻¹ .
Bispan®	Human	Binex Co. Ltd, Busan, S. Korea www.bi-nex.com	Tablet carrying spores (1.7 × 10 ⁷) of <i>B. polyfermenticus</i> SCD ^d [31,79].
Domuvar	Human	BioProgress SpA, Anagni, Italy http://www.giofil.it	Vial carrying 1 × 10 ⁹ spores of <i>Bacillus clausii</i> in suspension, labelled as carrying <i>B. subtilis</i> . No longer marketed [12].
Enterogermina®	Human	Sanofi Winthrop SpA, Milan, Italy www.automedicazione.it	Vial (5 ml) carrying 1 × 10 ⁶ spores of <i>B. clausii</i> in suspension. At least four different strains of <i>B. clausii</i> present and product originally labelled as carrying <i>B. subtilis</i> [7–14,19,141,184].
Esporafeed Plus®	Swine	Norel, S.A. Madrid, Spain	1 × 10 ⁹ <i>B. cereus</i> (CECT 953). Not licensed in the EU [43].
Flora-Balance	Human	Flora-Balance, Montana, USA www.flora-balance.com	Capsules labelled as carrying <i>Bacillus laterosporus</i> BOD ^d but containing <i>Brevibacillus laterosporus</i> BOD [6].
Lactipan Plus	Human	Istituto Biochimico Italiano SpA, Milan, Italy	Capsule carrying spores of <i>Bacillus subtilis</i> labelled as carrying 2 × 10 ⁹ spores of <i>Lactobacillus sporogenes</i> ^d [12].

Table 1 (continued)

Product	Target	Manufacturer	Comments/References
Lactopure	Human Animal feed	Pharmed Medicare, Bangalore, India http://www.pharmedmedicare.com	Labelled as <i>Lactobacillus sporogenes</i> ^d but contains <i>B. coagulans</i> [6].
Lactospore	Human	Sabinsa Corp., Piscataway, NJ, USA www.sabinsa.com	Labelled as carrying <i>Lactobacillus sporogenes</i> ^d but contains <i>B. coagulans</i> 6–15 × 10 ⁹ g ⁻¹ [6].
Liquallife [®]	Aquaculture	Cargill, Animal Nutrition Division www.cargill.com	Undefined <i>Bacillus</i> species [44].
Medilac	Human	Hanmi Pharmaceutical Co. Ltd., Beijing, China http://www.hanmi.co.kr	<i>B. subtilis</i> strain RO179 (at 10 ⁸ g ⁻¹) in combination with <i>Enterococcus faecium</i> [6].
Nature's First Food	Human	Nature's First Law, San Diego, CA, USA http://www.rawfood.com	42 species listed as probiotics including: <i>B. subtilis</i> , <i>B. polymyxa</i> ^d , <i>B. pumilus</i> and <i>B. laterosporus</i> ^d [6].
Neoform BS 10	Animals	Sanofi Sante Nutrition Animale, France	2 strains of <i>B. clausii</i> (CNCM MA23/3V and CNCM MA66/4M). Not licensed in the EU [185].
Neolactoflorene	Humans	Newpharma S.r.l., Milan, Italy	Mixture of lactic acid bacteria inc. <i>L. acidophilus</i> , <i>B. bifidum</i> and <i>L. sporogenes</i> ^d <i>L. sporogenes</i> at 3.3 × 10 ⁵ CFU g ⁻¹ whose valid name is <i>B. coagulans</i> is mislabelled and is a strain of <i>B. subtilis</i> [179].
Paciflor [®] C10	Calves, poultry, rabbits and swine	Intervet International B.V. Wim de Körverstraat 35 NL-5831 AN Boxmeer (NL)	<i>B. cereus</i> CIP5832 ^b (ATCC 14893) 2 × 10 ⁸ –5 × 10 ⁹ spores per dose dependant on target species. Withdrawn from production in 2002 [18].
Pastylbio	Humans	Pasteur Institute of Ho Chi Minh City, Vietnam.	Sachets (1g) carrying 10 ⁸ spores of <i>B. subtilis</i> .
Primal Defense [™]	Humans	Garden of Life [®] http://www.thehomeostasisprotocol.com/mall/Primal-defense/article3.htm	14 bacterial components including <i>B. subtilis</i> and <i>B. licheniformis</i> .
Promarine [®]	Aquaculture-shrimps	Sino-Aqua company Kaohsiung, Taiwan www.sino-aqua.com	Carries 4 strains of <i>B. subtilis</i> [151].
Subtyl	Human	Mekophar, Pharmaceutical Factory No. 24, Ho Chi Minh City, Vietnam	Capsule carrying 10 ⁶ –10 ⁷ spores of a <i>B. cereus</i> species termed <i>B. cereus</i> var <i>vietnami</i> . Product labelled as carrying <i>B. subtilis</i> [12,19].
Toyocerin ^{®c}	Calves, poultry, rabbits and swine. Possible use also for aquaculture	Asahi Vet S.A., Tokyo (Head Off.), Japan http://www.asahi-kasei.co.jp	<i>B. cereus</i> var <i>toyoi</i> (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 ^c [41].

^a This list is probably not complete and new products are being introduced or updated continuously.

^b Paciflor[®] and Bactisubtil[®] are thought to carry the same strain of *B. cereus* but are labelled differently as IP 5832 (Institute Pasteur, Bactisubtil[®]) and CIP 5832 (Paciflor[®]).

^c Authorised by the EU for unlimited use.

^d Not officially recognised as a *Bacillus* species (www.bacterio.cict.fr).

^e Authorised by the EU on a provisional basis.

min K₂ and have anti-cancer properties [32–35]. The extensive use of this fermented food product in Japan and the widely held belief in its beneficial properties appears to support the concept of probiosis.

2.1.3. Therapeutic products

Bacillus probiotics are also being developed for topical and oral treatment of uremia. Kibow Biotech (Philadelphia, USA; www.kibowbiotech.com) is developing *B. coagulans* probiotics for the treatment of gastrointestinal infections based on a number of PCT patents (e.g., WO9854982). This concept is based, in part, on the ability of *B. coagulans* to secrete a bacteriocin, Coagulin, that has activity against a broad spectrum of enteric mi-

crobes [36] and published reports showing the beneficial effects of *Bacillus* probiotics on urinary tract infections [37].

2.2. Animal products

In Europe, by 1997, farming was the second largest consumer of antibiotics after the medical profession. Of this, almost one third were being used as feed supplements and the remaining two thirds being used for therapeutic applications. In 1997 avoparcin was banned for use in animals [38] followed, in 1999, by four further antibiotics (bacitracin, spiramycin, tylosin and virginiamycin) which were banned for use as feed supplements

in the EU following an assessment by the Scientific Committee on Animal Nutrition (SCAN) [39]. Only four antibiotics remain licensed for use in animal feed (bambermycin, avilamycin, salinomycin and monensin) and a complete ban on these is due to take effect in 2006 [2]. In the absence of antibiotic usage in animal feed good husbandry will become paramount, as well as a renewed interest in the development of animal vaccines. Other approaches are the use of prebiotics, probiotics and synbiotics. Prebiotics are non-digestible food ingredients that can stimulate the growth and metabolic activity of bacteria present in the colon [40] and synbiotics are a mixture of pre- and probiotics.

In the EU two *Bacillus* products are licensed for animal use, BioPlus[®] 2B and Toyocerin[®] (see Table 1) [41,42]. In the case of Toyocerin[®] this contains a strain of *B. cereus* var *toyoi* that has been deemed safe for animal use because of its failure to produce enterotoxins and its failure to transfer antibiotic resistance. On the other hand a number of *Bacillus* products have not been licensed or withdrawn completely, most notably, Paciflor[®] C10 that carried a toxin producing strain of *B. cereus* (CIP 5832) and was considered a risk to human health [18]. Similarly, Esporafeed Plus[®] failed to satisfy SCAN that the *B. cereus* strain contained in the product did not produce enterotoxins [43]. In addition, this *B. cereus* strain was found to carry a tetracycline resistance gene, *tetB*, within its genome. Since *tetB* is normally located on a transposon its capacity to transfer this gene could not be ruled out.

2.3. Aquaculture

The use of *Bacillus* species in aquaculture is probably unfamiliar to most researchers in the *Bacillus* community, but it is a field that is expanding rapidly in countries with intensive farming of fish, and particularly shellfish (e.g., SE Asia) [44,45]. Cultured shrimps and prawns are now the fastest growing food production sector, with the black tiger shrimp (*Penaeus monodon*) being one of the most profitable ventures. Larval forms of most fish and shellfish are particularly sensitive to gastrointestinal disorders because they are released into the environment at an early stage before their digestive tract and immune system has fully developed [46]. In intensive farming the detritus that accumulates in a rearing pond can promote the growth and proliferation of pathogens and can have catastrophic impact on the resulting harvest. Economic losses due to disease can be substantial for those countries depending heavily on aquaculture for income and in 1996 alone were greater than US\$ 3 billion. Probiotic supplements that can treat larvae would therefore have a substantial impact on these losses. Shrimps have a non-specific immune response and vaccination (even if feasible) can only provide short-term protection against pathogens.

Probiotic treatments on the other hand provide broad-spectrum protection. A number of commercial products carry *Bacillus* spores, for example, the biocontrol product Biostart[®] (see Table 1). There are three distinct uses of bacterial supplements in aquaculture, probiotics, biocontrol agents and as bioremediation agents. *Bacillus* spp. are being used as probiotics and as biocontrol agents since bacteria used for bioremediation are usually nitrifying bacteria and are used to degrade the detritus generated from fish and shellfish in rearing ponds. Biocontrol refers to the use of bacterial supplements that have an antagonistic effect on pathogens [44]. *Bacillus* species have been used as components of biocontrol products and often are composed of mixtures of *Bacillus* species (e.g., Biostart[®] and Licalife[®]; see Table 1) [44]. Other commercial products that have been developed include the single species probiotic products, Toyocerin[®] and Paciflor[®] 9 [45,47]. Intriguingly, both of these products carry strains of *B. cereus* that are used commercially in animal feed and it is not clear whether these products are still in use. One effective strategy being used in developing countries is the isolation of *Bacillus* species from shrimp ponds and then using these as commercial products, one example of which is the product PF used in shrimp feed and containing a *Bacillus* species labeled S11 [48].

3. The natural habitat of *Bacillus* species

Bacillus species are saprophytic Gram-positive bacteria common in soil, water, dust and air [49]. They are also involved in food spoilage (e.g., spoilage of milk by *B. cereus* strains [50]). These bacteria are considered allochthonous and enter the gut by association with food.

4. The gut as a habitat for *Bacillus* species

Since spores of *Bacillus* species can readily be found in the soil, one might assume that the live (vegetative) bacteria that produced these spores are also soil inhabitants. This, however, is proving an unfounded assumption and, of course, the ability of spores to be dispersed in dust and water means that spores can be found almost everywhere. So, where they are found does not indicate their natural habitat (for an interesting review on this subject see [49]). Careful examination of the literature reveals that *Bacillus* spore-forming species are commonly found in the gut of animals and insects and experimentally this is often demonstrated by faecal sampling. The presence of *Bacillus* species, whether as spores or vegetative cells, within the gut could arise from ingestion of bacteria associated with soil. However, a more unified theory is now emerging in which *Bacillus* species exist in

an endosymbiotic relationship with their host, being able temporarily to survive and proliferate within the GIT. In some cases though, the endosymbiont has evolved further into a pathogen, exploiting the gut as its primary portal of entry to the host (*B. anthracis*) or as the site for synthesis of enterotoxins (*B. cereus*, *B. thuringiensis*) [51].

4.1. Humans

Bacillus species are often identified in large numbers within the gut far above what might be expected if these species were derived from ingested plant matter. The dominant bacteria found in the small and large intestine are species of *Lactobacilli*, *Streptococci*, *Enterobacteria*, *Bifidobacteria*, *Bacterioides* and *Clostridia*, yet *Bacillus* species also exist here. For example, in a study of human faeces, *Bacillus* species (listed as *B. subtilis* and *B. licheniformis*) were isolated in numbers of between 5×10^3 and 5×10^6 CFU g⁻¹ faeces [52]. In comparison, this study identified *Bacterioides* spp. at between 10^{11} – 10^{12} CFU g⁻¹ and *Streptococci* at 10^3 – 10^{10} CFU g⁻¹. In another study, *B. subtilis* was identified in high numbers in both elderly persons and infants [53,54]. Other examples of *Bacillus* species that are known to be able to survive in the human GIT are two members of the *Bacillus cereus sensu lato* species group, *B. anthracis* and *B. cereus* [51]. In the case of *B. anthracis* ingestion of spores will lead to gastrointestinal anthrax following uptake of spores into the GALT followed by germination and subsequent proliferation and dissemination [55]. *B. cereus* is a well known cause of food poisoning producing two distinct types, a diarrhoea and an emetic type syndrome [50]. If sufficient numbers of spores are ingested this can lead to a short-term illness and the dose has been determined as 10^5 – 10^7 g⁻¹ of ingested food for the diarrhoea syndrome and 10^5 – 10^8 g⁻¹ for the emetic syndrome [56]. Both types of food poisoning result from the action of enterotoxins into the lumen of the GIT and the emetic type is due to the ingestion of preformed toxin [56,57]. *B. cereus* is frequently found in faecal samples [58,59] and the faecal abundance of *B. cereus* appears to fluctuate according to the diet and its presence in food products (e.g., rice, pasta and milk); so, at most, it may be a transitory resident of the gut microflora. *B. cereus* isolates (most probably *B. thuringiensis*) have also been recovered in the faeces of greenhouse workers working with, and exposed to, *B. thuringiensis* biopesticides [60].

4.2. Mammals

Using a mouse model fed with a controlled diet, analysis of 16S rRNA libraries of total genomic DNA repeatedly identified *Bacillus mycoides* (a member of the *Bacillus cereus sensu lato* species group) in samples of the small intestine [61]. *B. thuringiensis*, also a mem-

ber of the *Bacillus cereus sensu lato* species group, has been found in the faeces of wild animals in Japan and Korea [62,63]. One of the most unusual spore-formers found in the gut is *Metabacterium polyspora*, a large, anaerobic Gram-positive spore-forming bacterium found only in the guinea pig [64]. The life cycle of this bacterium is intimately coupled with its passage through the GIT, involving germination of spores in the small intestine and then binary division coupled with the formation of multiple spore progeny. Spores excreted in the faeces enter the guinea pig gut by coprophagia and, indeed, this bacterium cannot be cultured outside its host. Most likely, other new types of unculturable strains exist that exhibit a *M. polyspora*-type life cycle in other coprophagic animals.

4.3. Aquatic animals

There are numerous reports of *Bacillus* species being isolated from fish and crustaceans, as well as shrimps [44]. It is important to remember that *Bacillus* spp. will be found at the bottom of ponds, lakes and rivers and many fish, crustaceans and shellfish will ingest *Bacillus* from this organic matter. Even so, *Bacillus* species are recovered from the GIT of aquatic animals with remarkable ease. They have been isolated from fish, crustaceans, bivalves and shrimps [44] and have been found in the microflora of the gills, skin and intestinal tract of shrimps [65]. In this laboratory, we have identified at least 12 *Bacillus* species from the gut of shrimps (*Penaeus monodon*) found in commercial shrimp farms in Vietnam (unpublished data).

4.4. Insects

Members of the *Bacillus cereus sensu lato* species group are frequently found in invertebrates. *B. cereus* has been identified in the gut of numerous insects, including aphids, mosquito larvae and cockroaches [51,66] and in certain arthropods this organism exists in a special filamentous or 'Arthromitus' stage within the intestine [67]. *B. cereus* as well as *B. mycoides* in the vegetative form has also been found in abundance in the gut of the earthworm (cited in [51]). *B. anthracis* has been found in the faeces of tabanid flies (various horse and deer flies) and this is believed to help disseminate and transmit anthrax [68]. *B. thuringiensis* is considered an insect pathogen due to its unique ability to produce large crystal protein inclusions during sporulation. These inclusions have biopesticide activity and are active against larvae from different insect orders including *Lepidoptera*, *Diptera* and *Coleoptera* [51]. *B. thuringiensis* does not grow in the soil, yet its presence there is believed to arise from insect deposition and it has been shown to proliferate in the earthworm gut [69]. It has been suggested that members of the *B. cereus sensu lato*

species group possess two life cycles, one where the bacteria live in a symbiotic relationship with their invertebrate host and a second life cycle where they can proliferate in a second invertebrate or vertebrate host [51]. Other *Bacillus* species found in the gut of insects include *B. licheniformis*, *B. cereus*, *B. sphaericus*, *B. circulans*, *B. megaterium*, *B. alvei* and *B. pumilus* [70–73]. As well as *B. thuringiensis* a number of other spore formers are insect pathogens that gain entry to the host via the GIT, these include *Paenibacillus larvae* (formerly *Bacillus larvae*) that infects domestic honeybees [74] and two species that produce parasporal crystals and are pathogenic to larvae of various *Coleoptera*, *Paenibacillus popilliae* (formerly *Bacillus popilliae*) and *Paenibacillus lentimorbus* (formerly *Bacillus lentimorbus*) [75].

5. The fate of ingested spores

What happens to spores following ingestion? Bacterial spores might be treated as a food and be broken down in the stomach and small intestine by the action of intestinal enzymes. Spores, though, are inherently robust bioparticles so it might be predicted that a large percentage survive the stomach, transit the GIT and are finally excreted in the faeces. Of course, this assumption is based on the notion that (i) most *Bacillus* species are facultative aerobes and so could not proliferate in the GIT, and (ii) most *Bacillus* species have no normal interaction with the GIT since they are soil organisms.

5.1. Transit kinetics

Experimentally it is possible to examine the fate of spores following ingestion. In humans this study has been performed using four volunteers who had been given a fixed dose of 10^5 *Bacillus stearothermophilus* [76]. For the first four days post-dosing the number of *B. stearothermophilus* CFU g^{-1} excreted in the faeces was maintained at a constant level after which counts dropped to insignificant levels by day 8. In a similar study, *B. stearothermophilus* was found to be present in the faeces for 10 days following initial dosing [77]. Interestingly, in this study the transit kinetics of *B. stearothermophilus* was similar to that of a *Lactobacillus* probiotic bacterium *L. plantarum* which showed evidence of colonization or, at least, retention in the GIT. It should be noted that these experiments counted spores only at the time of faecal sampling (as CFU g^{-1}) but did not show the total counts of spores excreted. Even so, these studies revealed that the transit time (or longevity) of *B. stearothermophilus* in the human GIT was 8–10 days, somewhat longer than the experimentally calculated transit time of a solid marker in the gut [78]. In a third human study 10 volunteers were given two tablets containing 1.667×10^7 spores of *Bacillus*

polyfermenticus SCD ([31]; note this is not valid species) once a day for 14 days [79]. In this work counts of *B. polyfermenticus* SCD were still detectable 4 weeks after the final dosing (e.g., 10^3 CFU g^{-1} faeces at week 6). These results differ substantially from those of *B. stearothermophilus* since if spores have no interaction with the GIT and simply pass through then we would expect to see no counts of *B. polyfermenticus* SCD after 22–24 days (based on the *B. stearothermophilus* studies described above). Although the dosing regime was different two explanations can be proposed, first, the difference may be species-specific and perhaps *B. polyfermenticus* SCD spores are somehow able to adhere to the gut lining retarding their transit. Alternatively, spores could be proliferating within the GIT and able to temporarily colonise. To proliferate, spores must be able to germinate and then replicate. The transit of the probiotic product Paciflor[®] C10 (*B. cereus* CIP 5832) has also been determined in dogs given 10^6 spores g^{-1} of meal [80]. Spores and vegetative cells were first detectable in the faeces 24 h after ingestion and could not be detected after 3 days showing no evidence of colonization. Studies examining the fate of spores in murine models are discussed below.

5.2. Spore germination and proliferation

The first studies indicating that spores could germinate in the GIT came from experiments using ligated ileal loops of rabbits [81]. More thorough studies in vivo have used a murine model. Here different doses of spores (from 10^8 to 10^{10}) of the *B. subtilis* strain PY79 ([82]; derived from the 168 type strain) were administered to groups of inbred (Balb/c) or outbred mice [83]. In each case mice were housed individually and by using gridded cage floors total faeces could be collected at 1–2 day intervals. These studies showed that the first spore counts were detectable in the faeces 3 h post-dosing yet, more importantly, after 18 h more spores had been excreted than were administered. By 5–7 days no significant spore counts could be detected yet the cumulative counts showed an increase in total CFU by as much as 6-fold. Since the total counts was greater than the administered dose the only explanation was that the spores had germinated, proliferated to some extent and then re-sporulated. This seemed at first, implausible, since no direct evidence had yet been given that *B. subtilis* spores could germinate. Moreover, *B. subtilis* is considered a facultative aerobe so how could it survive in the anoxic conditions in the GIT? Recent studies though, have shown that under appropriate conditions ‘aerobic’ strains of *B. subtilis* can grow anaerobically if able to utilize nitrate or nitrite as an electron acceptor or by fermentation in the absence of electron acceptors [84]. The finding that *B. subtilis* spores could germinate should not be so surprising. Firstly, the spore is a dor-

mant life form and presumably the upper region of the small intestine would be rich in nutrients that could induce germination, a process that does not require *de novo* protein synthesis. Second, as already mentioned, some *Bacillus* spore formers are already known to germinate and proliferate in the GIT, most notably *B. cereus* (see below). What was surprising was that the germinating spore could outgrow, replicate and re-sporulate. It is also likely that the GIT is not strictly anoxic, especially in the small intestine, and could contain a sufficient microenvironment for growth of *B. subtilis*. Supporting this, some microaerophilic bacteria such as *Heliobacter* and *Campylobacter* can grow readily in the GIT. *B. subtilis* has not been the only spore forming species shown to be able to germinate. Recent studies have also shown germination of *B. cereus* var *toyoi*, the commercial strain present in Toyocerin[®], in poultry and in pigs [85,86]. In these studies rapid germination in the upper intestine in both animal species was observed reaching levels of 90% of the administered spore dose in the crop of broiler chickens. Interestingly, this work also showed that sporulation could readily occur in the small intestine by dosing piglets with 10^8 vegetative cells and showing that after 22 h over 10^7 spores g^{-1} of digesta could be recovered. Similar results were obtained in the same study using broiler chickens. These results show that *B. cereus* var *toyoi* vegetative cells are intrinsically resistant to both gastric juice and to bile salts. Interestingly, similar studies using *Lactobacillus* (*L. plantarum* NCIMB 8826, *L. fermentum* KLD) and *Lactococcus* (*Lc. Lactis* MG 1363) probiotic strains showed that, at most, only 7% of an oral dose survived transit to the small intestine [77]. *B. subtilis* var. *natto* has also been shown to germinate in the GIT of mice [34].

Conclusive proof that *B. subtilis* spores do indeed germinate was made using a molecular method [87]. Two chimeric genes were made by fusing the 5' region of the *ftsH* gene of *B. subtilis* to the *lacZ* gene of *Escherichia coli*. The *ftsH* gene is expressed only during vegetative cell growth and so *ftsH-lacZ* mRNA could only be produced in the vegetative cell. Spores carrying *ftsH-lacZ* were used to dose mice and the presence of *ftsH-lacZ* cDNA identified by RT-PCR analysis from total RNA extracted from homogenized sections of the small intestine. These studies demonstrated spore germination in the jejunum. Similar studies using a *rrnO-lacZ* chimera revealed germination in the ileum as well [87]. While spore germination has been proven, the level of *B. subtilis* spore germination is not known, although extrapolative studies suggest this is probably less than 1% of the inoculum [87]. Interestingly, in studies counting spores excreted in faeces an increase in numbers was not always seen, suggesting that the physiological conditions (e.g., diet) of the host might affect germination and/or proliferation.

5.3. Resistance to intestinal fluids

In vitro studies have shown that strains of *B. coagulans* cells are sensitive to simulated gastric fluid (SGF; pH2-3) but tolerant to bile salts at 0.3% with a MIC of greater than 1% [88]. *B. subtilis* has been examined in vitro in two studies. The first showed that *B. subtilis* cells were extremely sensitive to SGF and bile salts (0.2%) with an almost complete loss of viability in 1 h [89]. A further study has shown the MIC of bile salts for *B. subtilis* to be 0.4% and for two probiotic strains, *B. cereus* IP5832 (Bactisubtil[®]) and *B. clausii* (Enterogermina[®]) as 0.2% and <0.05%, respectively [13]. By contrast, spores of *B. subtilis* have been shown to be fully resistant to SGF and bile salts although germination of *B. subtilis* spores was partially inhibited by bile salts [89]. An interesting and unexpected study has also shown that not all spores are resistant to SGF and bile salts. Specifically, spores of the *B. cereus* strain used in the commercial product Biosubtyl were shown to be extremely sensitive to SGF and also to bile salts whereas spores of other *B. cereus* strains were completely resistant [19]. One explanation for these unexpected results is that spores may be subject to acid-induced activation of spore germination (as opposed to heat-induced germination [90,91]). Germination of spores is an extremely rapid process so acid-induced germination could generate a large population of vegetative cells that are killed by SGF. These same spores were also sensitive, but less so, to bile salts.

An in vivo study showed that after dosing mice with 2×10^8 *B. subtilis* spores almost all spores could survive transit across the stomach and could be recovered from the small intestine [89]. In contrast, vegetative cells had almost no survival in the stomach and only a tiny fraction were able to survive transit (<0.00016% of the administered dose), confirming in vitro studies with *B. subtilis* (see above), but in contrast to *B. cereus* var *toyoi*. To account for those survivors one must assume either that they are associated with food matter or had clumped in such a way as to survive transit.

These studies appear to show that, with a few exceptions, vegetative *Bacillus* cells are sensitive to conditions within the GIT and that the stomach, in particular, presents a formidable barrier. Spores on the other hand are unaffected. It is important to remember though, that the gastric physiology of the mouse will be different from humans (e.g., a higher stomach pH) so any predictions must be tentative. If spores do indeed germinate and sufficient evidence now exists to show this does occur, then to survive and proliferate, the cell must find a way to escape the toxicity of the luminal fluids of the GIT. Passage through the GIT from the small intestine would dilute the toxic effects of bile salts but would in turn deliver cells into the anaerobic environment of the colon. Possibly, the shielding effect of food or clumping is suf-

ficient to provide some protection. Alternatively, perhaps, adhesion to the gut mucosa and the formation of mixed biofilms with the gut microflora could provide a temporary niche. Ultimately, the logical pathway for spore formers to take under conditions of extreme stress would be re-sporulation and this has now been shown to occur and seems a plausible strategy for surviving transit through the GIT.

5.4. Colonisation

Currently, there appears to be no compelling evidence that non-pathogenic, spore-forming bacteria permanently colonise the GIT and this ability, if any, may depend on the host, the specific spore-forming species, and other physiological and dietary factors. Even with pathogenic strains of *B. cereus* the infection is temporary (approx. 24 h) and *B. cereus* is shed completely after 24–48 h [50,57]. It is worth remembering that our knowledge of *Bacillus* is far from complete and no dedicated studies have been made to examine *Bacillus* species in the gut of animals, and most studies have examined specific strains or pathogens. It can not be ruled out that new colonizing *Bacillus* species or strains have yet to be identified.

However, at least two studies using chicks has shown that after being given a single dose of spores (2.5×10^8) *B. subtilis* can persist for up to 36 days in the avian intestine [92,93]. Examination of the transit time of *Bacillus* probiotic strains in the mouse gut has shown that, following a single dose of spores, the levels of viable counts detectable in faeces after 15 days was barely significant [19]. Interestingly though, when compared in parallel to a laboratory strain of *B. subtilis* (a derivative of the 168 type strain), which was completely shed within 6 days, all probiotic *Bacillus* strains showed greater retention within the mouse gut, suggesting that they could persist longer. How this could occur is not yet known but might arise from adhesion of the vegetative cell to the mucosal epithelium as is known to occur with pathogenic *B. cereus*. In *B. cereus* the crystalline S-layer that forms the outermost layer of the vegetative cell has been implicated in adhesion as well as resistance to phagocytosis [57,94]. At least 18 species of *Bacillus* have been documented as possessing S-layers [95]. The S layer has not been shown to have a role as a protective coat since they carry pores large enough to allow the transit of enzymes, so a role in evading phagocytosis or adhesion cannot be ruled out. Relatively little is known about the detailed morphology of spores and their adhesive properties in vivo, but in most *Bacillus* species (although not *B. subtilis*) the entire endospore is contained within a loose sack known as the exosporium [96]. The exosporium has no unified structure but it can be physically removed without harm to the spore; and its composition and appearance under electron-

microscopy vary considerably between species [97]. One role for the exosporium could be in adhesion [98]. Another structure that could be involved in adhesion is a novel pilus structure found on the surface of the spore in strains of *B. cereus* and *B. thuringiensis* [98–102]. Pili are not present in the vegetative cell of *B. cereus* [102] so this structure could be important for initial adhesion to the gut epithelium. Interestingly, this structure is not restricted to potentially virulent species. New isolates of *B. clausii* obtained from the gut of poultry have been identified that also possess spore pili [103].

In the case of *B. cereus*, spores of different strains have been shown to adhere to several types of surface and *B. cereus* strains have been shown to be more hydrophobic than other *Bacillus* spp. [104]. A recent study has shown that binding to Caco-2 (human epithelial) cells was found to be directly proportional to the hydrophobicity of spores themselves and the greater the hydrophobicity of the spore, the greater its adhesive properties [105]. If spores of other *Bacillus* spp. also have some ability to adhere to the mucosal epithelium based on their hydrophobicity, then it might explain the varied transit times of different probiotic strains shown in the study of Duc et al. [19]. A further consideration is the formation of biofilms on the mucosal epithelium. Most of the gut microflora exists in mixed biofilms attached to the mucosal epithelium or to food particles [106,107], and *B. subtilis* has been shown to produce multicellular structures and biofilms [108]. These robust films have aerial structures, referred to as fruiting bodies, that have been shown to act as preferred sites for spore formation [109]. These studies on the interaction of *Bacillus* spp. with surface layers mimicking their natural environment show how little is still known about spore formers in their natural environment.

5.5. Dissemination and intracellular fate

An important aspect of evaluating the safety of a probiotic bacterium is whether it can cross the mucosal epithelium, disseminate to target tissues and organs and even proliferate. One study has recently addressed this using spores of a laboratory strain of *B. subtilis* [110]. Inbred mice were given 10^9 spores in each daily dose for 5 consecutive days. Low, yet significant viable counts (representing mostly spores) were recovered in the Peyer's Patches and mesenteric lymph nodes. Although no dissemination to deep organs (liver and kidneys) was observed these results did show that a proportion of spores must have crossed the mucosal barrier. *B. subtilis* spores are approximately 1.2 μm in length and so are of sufficient size to be taken up by M cells that are localised in the mucosal epithelium of the small intestine and then carried to the Peyer's Patches before transportation to the efferent lymph nodes. The Peyer's Patches

are rich in antigen-presenting cells, particularly dendritic cells that are effectors of Th1 and Th2 cellular responses. An *in vitro* study has shown that murine macrophages (a RAW264.7 cell line) cultured in the presence of *B. subtilis* spores could efficiently phagocytose spores [111]. Surprisingly, these studies also demonstrated that spores could germinate within the phagosome and initiate vegetative gene expression as well as protein synthesis. Germinated spores, though, failed to grow and divide and after approximately 5 h were destroyed, presumably by fusion of the phagosome with a lysosome. These results offer striking analogies with *B. anthracis* that exploits phagocytosis to gain entry into a host cell. *B. anthracis* germinates within the phagosome and can proliferate and secrete toxins which lead to cell lysis [112–114]. Unlike *B. subtilis* the *B. anthracis* vegetative cell is encased in a capsule that protects it from the toxic intracellular environment. It was proposed that intracellular spore germination may be induced by the phagocytic cell as a first step in destroying the bacterium, and the phagocytic cell possibly provides an appropriate signal to stimulate germination [111]. Interestingly, genes involved in germination appear to be remarkably conserved amongst *Bacillus* species, so the germination process per se is likely to be similar between species [115]. This study is important because it shows that ingested spores delivered to the small intestine in large numbers can interact with the gut-associated lymphoid tissue (GALT). Interaction with the GALT, as will be discussed below, is an efficient mechanism to stimulate the immune system and could provide a mechanism for probiosis.

6. In vivo studies addressing the efficacy of *Bacillus* probiotics

6.1. Human studies

There are few published reports of clinical trials. One study has examined the effect of *B. clausii* (reported as *B. subtilis* ATCC 9799 and the species found in Enterogermina[®]) spores on 80 elderly patients with slow or static urinary flow [37]. This randomized and placebo-controlled study used patients treated for 6 months with two vials of Enterogermina[®] daily. In the final 2 months of treatment there was a statistically significant reduction ($P < 0.05$) in the number of patients with positive urine cultures (i.e., containing $>10^5$ bacterial counts; typically species of *Klebsiella*, *Proteus*, *Shigella*, *Pseudomonas* and *E. coli*). *B. coagulans* (reported incorrectly as *Lactobacillus sporogenes*) was used in a nonrandomized study of 17 hyperlipidemic patients [116]. Two tablets (each containing 3.6×10^8 spores) were given to patients three times per day for twelve weeks. In this study, which unfortunately did not carry dietary controls,

reductions in total cholesterol were observed. In a recent trial *B. coagulans* has been used successfully to prevent antibiotic-associated diarrhoea in children [117].

6.2. Animal studies

One day old chicks dosed orally with a single dose of spores (2.5×10^8) of a laboratory strain of *B. subtilis* showed greater resistance to the avian pathogen *Escherichia coli* O78:K80 when challenged 24 h following dosing [92]. These studies showed a significant reduction in the colonization of the spleen, liver and caeca. Intriguingly, *E. coli* O78:K80 infection and colonization could not be suppressed when birds were challenged 5 days after initial dosing with *B. subtilis*. In a similar study, the same laboratory strain of *B. subtilis* was found to suppress colonization and persistence of *Salmonella enteritidis* and *Clostridium perfringens* in chicks [93]. Interestingly, *B. subtilis* was found to persist in the avian intestine for 35 days suggesting that it may briefly colonise the GIT and appears to be supported by studies showing extensive spore germination and colonization of broiler chickens by *B. cereus* var *toyoi* [85]. *B. subtilis* var. *natto*, the bacterial component of the fermented food product Natto has been shown to improve feed conversion efficiency and reduce abdominal fat of broiler chickens [118]. These animals all showed a reduced ammonia concentration, which was proposed to activate intestinal function including villus height and enterocyte cell area. Reduced ammonia concentrations have also been observed in probiotic-fed chickens and pigs fed with *B. cereus* [119,120] and these low ammonia concentrations have been shown to stimulate germination of *B. cereus* spores [121]. Moreover, reduced ammonia, by increasing the total surface area of the gut lumen could increase nutrient absorption and might provide one explanation of probiosis. *B. cereus* var *toyoi* has also been shown to increase abdominal fat in the Japanese quail (*Coturnix japonica*) [122]. The efficacy of the recently withdrawn probiotic product, Paciflor[®] C10 containing *B. cereus* CIP 5832, was shown to enhance the health status of sows and their litters [123]. Piglets receiving the probiotic (85 g ton⁻¹ of feed) showed a reduced incidence of scour (diarrhoea) and reduced mortality and a general increased feed conversion ratio of Paciflor[®]-treated piglets. *B. licheniformis* spores and *B. cereus* var *toyoi* have been shown to reduce the incidence and severity of post-weaning diarrhoea syndrome in piglets [124]. Animals displayed a gain in weight and enhanced feed-conversion efficiency. Additional studies have shown that pigs receiving *B. cereus* var *toyoi* had more pronounced intestinal villi similar to those described above for poultry dosed with *B. subtilis* var *natto*. *B. cereus* var. *toyoi* was one of three probiotic species evaluated for their effect on edema disease caused by ETEC in piglets and was found, as were the

other non-*Bacillus* probiotics, to have no effect on the disease [125].

In ruminants the digestive tract is more complex than in monogastric animals with food first entering the rumen before being passed to the reticulum (the second forestomach) and then to the abomasum (true stomach). Calves acquire a GI microflora after birth and as solid feed is consumed the microbial population of the rumen increases and diversifies. Probiotic bacteria are thought to be beneficial in rapidly establishing the rumen microflora and the more rapid this process the faster the transition from liquid to solid feed. In turn, this reduces the probability of gastrointestinal problems such as scouring (a diarrhoeal syndrome often caused by ETEC bacteria). Few studies have been undertaken to evaluate the effect of *Bacillus* probiotics in feed. However, one study has reported positive effects on feed conversion efficiency in calves fed with a *B. subtilis* strain (used in the commercial product BioPlus 2B[®]) in the first month post-weaning [126].

6.3. Fish and shrimps

One of the problems associated with evaluating *Bacillus* products (or indeed any probiotic product) for aquaculture is determining whether the observed effect is due to the action of the bacterium on the host gut or due to an indirect effect on water quality or antagonism of external pathogens [44]. Regardless, sufficient evidence suggests that adding *Bacillus* as spores or vegetative cells to rearing ponds has a beneficial effect. Toyocerin[®] has been used as a probiotic feed for Japanese eels and shown to reduce infection and mortality by *Edwardsiella* spp. [47]. *Bacillus* spores have been shown to increase the survival and production of channel catfish [127]. A strain of *B. subtilis* has been isolated from the common snook and it was shown that introduction of this isolate, as spores, into rearing water eliminated *Vibrio* species found in the larvae of snook [128]. *Bacillus* species appear to show most promise in prevention of *Vibrio* infections that are a major threat in intensive shrimp farming. Addition of *Bacillus* cells (not spores), selected on the basis of their ability to produce antibiotics against *Vibrio* species, to rearing ponds has been shown to decrease the numbers of *Vibrio* species in pond sediments as well as to increase prawn survival [129]. This study also illustrated the problem of determining whether the *Bacillus* species was directly involved or whether it improved water quality by degrading organic matter in pond sediments. The introduction of *Bacillus* spp. in the immediate proximity of pond aerators has been shown to significantly reduce chemical oxygen demand and lead to an increased shrimp harvest and this strategy has led to the development of some commercial products such as Biostart[®] [44]. A *B. subtilis* isolate, BT23, isolated from shrimp culture ponds has been

tested for its activity against *V. harveyi*, a shrimp pathogen, both in vitro and in vivo [130]. A cell-free extract of BT23 was shown to inhibit the activity of various *Vibrio* species using an agar diffusion assay. By co-culturing *V. harveyi* with *B. subtilis* BT23, growth of *V. harveyi* was inhibited and cell-free extracts of BT23 were bacteriostatic. In a challenge model the mortality of *V. harveyi* infection was significantly reduced by the presence of BT23 in tank water. These very simple experiments appear to show clear probiotic properties by *B. subtilis* BT23. Unfortunately in these experiments no attempt was made to define the inoculum as spores or as vegetative cells. A similar experimental rationale has been made using a *Bacillus* species (not defined) termed S11, isolated from the soil sediments of shrimp ponds [131]. In a challenge test *Penaeus monodon* treated with S11 vegetative cells showed 100% survival compared to a control that exhibited 26% survival. In a more extensive study this probiotic was shown to stimulate the shrimp immune system, to reduce shrimp mortality when animals were challenged with *V. harveyi*, and to be more effective when given to juvenile shrimps [132].

7. Mechanisms for probiosis

7.1. Immune stimulation

Stimulation of the immune system, or immunomodulation, is considered an important mechanism to support probiosis. A number of studies in humans and animal models have provided strong evidence that oral administration of spores stimulates the immune system. This tells us that spores are neither innocuous gut passengers nor treated as a food. As already stated, a small proportion of *B. subtilis* spores have been shown to disseminate to the primary lymphoid tissues of the GALT (Peyer's Patches and mesenteric lymph nodes) following oral inoculation [110] and in vitro studies have shown that phagocytosed spores can germinate and express vegetative genes but are unable to replicate [111]. Following oral dosing, anti-spore IgG responses could be detected at significant levels. Anti-spore IgG and secretory IgA (sIgA) could be produced by a normal process of antigen uptake by B cells. Detailed analysis of the subclasses showed IgG2a to be the initial subclass produced and this is often seen as being indicative of a type 1 (Th1) T-cell response [133–137]. Th1 responses are important for IgG synthesis but more importantly for CTL (cytotoxic T lymphocyte) recruitment and are important for the destruction of intracellular microorganisms (e.g., viruses, *Salmonella* spp.) and involve presentation of antigens on the surface of the host cell by a class I MHC processing pathway. Support for Th1 responses has been provided by the analysis of cytokines in vivo that showed synthesis of IFN- γ and TNF- α in the

GALT and secondary lymphoid organs when spores of *B. subtilis* or *B. pumilus* were administered to mice [89,111]. IFN- γ is an effector of cellular responses and could have been produced by an innate immune response probably including Natural Killer (NK) cells. Similar studies have shown that orally administered *B. subtilis* leads to a rapid induction of interferon production by mononuclear cells in the peripheral blood, which stimulated the activity of both macrophages and NK cells [138]. A number of other studies have shown *ex vivo* synthesis of IFN- γ in rabbits or mice following dosing with *B. clausii* spores of the Enterogermina[®] product [139,140]. In a recent study vegetative cells of the four Enterogermina[®] *B. clausii* strains was shown to induce IFN- γ synthesis in murine spleen cells [141]. Interestingly, all *B. clausii* strains induced proliferation of CD4+ T cells in the presence of irradiated APC spleen cells and the peptidoglycan component of the cell wall is one component that could be involved in immunomodulation [142]. This is in agreement with studies using human mononuclear cells that showed that vegetative cells, but not spores, could stimulate mitogenic-induced lymphocyte proliferation *in vitro* [143]. *Bacillus firmus* vegetative cells have been shown to stimulate the proliferation of human peripheral blood lymphocytes *in vitro* [144]. In this study *B. firmus* was shown to promote differentiation of B lymphocytes to Ig producing and secreting cells and was shown to be significantly more potent than other *Bacilli* tested (*B. subtilis*, *B. coagulans*, *B. megaterium*, *B. pumilus*, *B. cereus* and *B. lentus*).

Another study involved a randomized trial of 30 elderly patients who were given *B. clausii* spores of Enterogermina[®]. Lymphocyte subsets were determined from peripheral blood mononuclear cells and a significant increase in B lymphocytes bearing membrane IgA was observed but not unrestricted proliferation of all B lymphocytes [145]. These results indicate that orally administered spores may be interacting with the GALT and priming B lymphocytes for IgA synthesis. An interesting study has shown that *B. subtilis* in combination with *Bacteroides fragilis* promoted development of the GALT in rabbits and led to the development of the pre-immune antibody repertoire [146]. Interestingly, neither species alone could induce GALT development, so this cannot be an antigen-specific immune response. Furthermore, at least one stress protein, YqxM, secreted from *B. subtilis* was shown to be required for GALT development.

On a more cautionary note, *in vitro* studies have shown that the proinflammatory cytokine IL-6 was produced in macrophages cultured with *B. subtilis* or *B. pumilus* spores [19]. Proinflammatory responses cannot necessarily be considered a beneficial feature of a probiotic since they have been linked to a number of autoimmune diseases such as inflammatory bowel diseases including ulcerative colitis and Crohn's disease [147].

Invertebrate immune systems have two components, first, humoral defenses, such as antibacterial activity, agglutinins, cytokine-like factors and clotting factors, and second, cellular defenses such as hemolymph clotting, phagocytosis, encapsulation and the prophenoloxidase system [148,149]. No evidence of antibody synthesis has yet been shown. In commercially farmed shrimps, an undefined *Bacillus* species, *Bacillus* S11, has been shown to stimulate the primitive immune system of *Penaeus monodon* [132]. *Bacillus* S11 cells were shown to increase phenoloxidase as well as antibacterial activity (against the shrimp pathogen *V. harveyi*) in shrimp hemolymph. *Bacillus* S11 was also shown to increase the levels of phagocytosis of hemocytes derived from hemolymph compared to control shrimps and levels were increased further after challenge of shrimps with *V. harveyi*. As with vertebrate studies that show cell wall peptidoglycan to be a potential immunogen, in shrimps, peptidoglycan has also been shown to stimulate granulocytes leading to higher levels of phagocytosis [150].

7.2. Synthesis of antimicrobials

The production of antimicrobials by probiotics is considered one of the principal mechanisms (microbial interference therapy) that inhibit pathogenic microorganisms in the GIT. *Bacillus* species produce a large number of antimicrobials (see [151]). These include bacteriocins and bacteriocin-like inhibitory substances (BLIS) (e.g., Subtilin and Coagulin) as well as antibiotics (e.g., Surfactin, Iturins A, C, D, E, and Bacilysin). Some *Bacillus* species contained in commercial products are known to produce antimicrobials. Of the two *Bacillus* strains in the product Biosporin[®] (Table 1) *B. subtilis* 3 has been shown to produce a heat-stable and protease-resistant isocoumarin antibiotic, aminocoumarin A [26]. This antibiotic was active against *Staphylococcus aureus*, *Enterococcus faecium*, *Shigella flexneri*, *Camphylobacter jejuni* as well as *Heliobacter pylori*. *B. clausii* strains in Enterogermina[®] have been shown to produce antimicrobials with activity against Gram-positive bacteria [19,141] and *B. polyfermenticus* SCD carried in the S. Korean product Bispan has been shown to produce a protease-sensitive and heat-labile bacteriocin, polyfermenticum, with activity against Gram-positive bacteria [31]. *B. subtilis* strains carried in the commercial products Promarine[®] and Bio Plus 2B[®] (Table 1) have also been shown to produce antimicrobials [151]. Probiotic strains of *B. coagulans* are found in a number of commercial products often mislabeled as *Lactobacillus sporogenes* (see Table 1). *B. coagulans* produces coagulin, a heat-stable, protease-sensitive BLIS with activity against Gram-positive bacteria [36]. *B. subtilis* var. *natto* has also been shown to inhibit the growth of *Candida albicans* [152] in the intestinal tract and a surfactin has been identified with activity against yeast [153]. The effect of

these antimicrobials in vivo is not understood and it cannot be assumed that effects seen in vitro can be mimicked in the host GIT. To illustrate this Hosoi et al. [34] dosed mice with *B. subtilis* var *natto* spores and showed that this promoted growth of *Lactobacillus* under some dietary conditions but decreased counts under others [35]. The microenvironment of the GIT is extremely complex and is subject to dietary and physiological conditions that influence the formation of biofilms on the gut epithelium. The ability of ingested probiotic bacteria to influence this microbiota is therefore going to be subject to a large number of factors that, in turn, influence its ability to survive and secrete antimicrobials.

7.3. Other mechanisms

The competitive exclusion (CE) concept is a term mostly used in the poultry industry and refers to the ability of orally administered bacteria to stimulate the host's resistance against infectious disease [154,155]. Different mechanisms have been proposed for CE agents including competition for host mucosal receptor sites, secretion of antimicrobials, production of fermentation by-products, such as volatile fatty acids, competition for essential nutrients and stimulation of host immune functions. This concept is mentioned here because it overlaps with probiosis but is often reported as a separate mechanism in poultry studies. In the poultry industry a number of products have been used that carry poorly defined mixtures of microorganisms, some carrying *Bacillus* species, and these have been shown to be beneficial to the host [119,156].

Bacillus species (*B. subtilis*, *B. firmus*, *B. megaterium* and *B. pumilus*) have recently been shown to convert genotoxic compounds to unreactive products in vitro and this has been proposed as a probiotic mechanism, if this could occur in the intestine [157]. It is generally accepted that maintaining the correct balance of commensal bacteria in the GIT is important to a number of gastrointestinal disorders such as diarrhoea, inflammatory bowel disease (Crohn's disease and ulcerative colitis) as well as colorectal cancer [158]. At least one study has shown that oral administration of *B. subtilis* var. *natto* in mice influenced the faecal microflora, specifically *Bacteroides* and *Lactobacillus* species, and that this depended upon the diet [34]. In these studies it was shown that the numbers of *Lactobacillus* spp. decreased when mice were fed with an egg white diet, but stabilized when the diet was supplemented with *B. subtilis* var. *natto* spores. Using a casein diet, however, the numbers of *Lactobacillus* spp. were unchanged when supplemented with spores, although the numbers of *Bacteroidaceae* increased. Interestingly, no change was seen when autoclaved spores were used, suggesting that the effect seen might be due to germinating spores. This work indicated that *B. subtilis* var. *natto* could be bene-

ficial in maintaining the natural microflora. Understanding the complexities of the diet and its affect on probiosis in appear daunting, although related studies in chickens and pigs provide supporting data [159].

8. The Safety of *Bacillus* products

The use of any probiotic whether for human or animal use should raise questions over safety, since the product is consumed in large quantities on a regular basis. For human use the primary concern is whether the bacterial species is safe to ingest and whether GMP conditions have been used in production. For animal use the concern is whether using the probiotic in animal feed increases the risk of inter-species transfer of antibiotic resistance genes. The Food and Drug Administration of the United States of America has not, as yet, granted any probiotic product GRAS (Generally Regarded As Safe) status although *Bacillus* species do carry GRAS status for specific industrial applications (e.g., enzyme production) [6].

8.1. Infections associated with *Bacillus* species

B. anthracis and *B. cereus* are known pathogens. *B. anthracis* will not be discussed here, nor will coverage be made of the voluminous reports documenting local, deep-tissue and systemic infections in immunocompromised patients and incidental reports of *Bacillus* species being isolated from hospital infections. Similar reports can be found for members of a number of bacterial genera. Reports detailing these infections can be found elsewhere (see [6,7,42,160]). *B. cereus* is worth summarizing, since strains of this species are in current use as a probiotic. *B. cereus* strains can produce either a diarrhoea-type disease or an emetic-type disease [50,56]. In the diarrhoea syndrome, the disease is produced by ingestion of spores in contaminated foodstuffs, germination of spores in the GIT and secretion of one of up to six enterotoxins, Haemolysin BL (Hbl), Non-haemolytic enterotoxin (Nhe), Enterotoxin T (BceT), Enterotoxin FM (EntFM) and Enterotoxin K (EntK). In the emetic syndrome, illness is caused by ingestion of the pre-formed emetic toxin, Cereulide. The severity of the diarrhoea syndrome is probably linked to the number of enterotoxins produced. It has been shown that not every strain of *B. cereus* carries all enterotoxin genes, and in some cases none at all [161]. For Nhe and Hbl the active toxin is composed of three subunits, each encoded by separate genes. Intriguingly, some *Bacillus* strains have been shown to carry one or more of these genes but not all. For example a strain of *B. sphaericus* (KD18) was found to contain the *hblA* and *hblD* genes but not *hblC* [161]. The commercial *B. cereus* product Bactisubtil[®] was found to carry the *nheB* and *nheC* genes but not *nheA* and did not produce the Nhe enterotoxin [19].

B. thuringiensis, which is closely related to *B. cereus*, is used as a biopesticide and has been implicated in cases of gastroenteritis in workers using this horticultural agent [60,162]. Strains of *B. thuringiensis* have also been shown to produce enterotoxins [60,163].

B. licheniformis has been reported in cases of food-borne diarrhoeal illness, toxin production and infant mortality [164]. *B. subtilis* has been implicated in food-borne illnesses, with vomiting being the most common symptom [165]. A recent study has shown that at least one *B. subtilis* strain carries all three genes required to produce the Hbl enterotoxin normally produced in *B. cereus* [161]. Therefore, even *B. subtilis* must be treated with caution and any use of it as a probiotic must follow a complete evaluation of virulence factors.

In cases of food-borne illnesses, especially diarrhoea, difficulties exist in identifying the causative agent. Since many probiotics are used to treat diarrhoea this can produce misleading conclusions, as illustrated in a recent study. Kniehl et al. [166] reported on three cases of diarrhoea where *B. cereus* was isolated from the stools of patients. Each isolate was confirmed as *B. cereus* strain IP5832 and was found to have originated from the probiotic Bactisubtil® (*B. cereus* IP5832) used to treat the diarrhoea. The Bactisubtil® *B. cereus* strain has been shown to produce diarrhoea-producing enterotoxins so the possibility could not be ruled out that the use of this product contributed to the diarrhoea syndrome.

8.2. Antibiotic resistance transfer

There are a number of important concerns over the use of probiotic bacteria relating to their ability to transfer and disseminate drug resistance genes [39,167]. Most importantly, their use in animal feed could create a reservoir of drug-resistance that is transferable to humans. Another scenario is the transfer of resistance genes to animal pathogens that can cross the species barrier and infect humans through food products. Finally, the release to the environment in faeces would enable an accumulation or drug-resistance genes that can survive in the absence of a selective pressure. One of the problems with probiotic usage in humans is that in some countries probiotics are prescribed as an adjunct to the antibiotic. This is a common practice in SE Asia where probiotic bacteria (*Bacillus* and *Lactobacillus* spp.) are used to limit the side effects of antibiotics and a number of products are marketed as carrying 'antibiotic-resistant probiotic bacteria' (including products licensed to European companies). In hindgut fermentors (e.g., humans and pigs) the major microbial populations reside in the large intestine (colon) and can consist of up to 10^{12} anaerobic bacteria ml^{-1} . In ruminants approximately 10^9 – 10^{11} anaerobic bacteria ml^{-1} are found in the rumen and also large populations of anaerobic bacteria (10^7 – 10^{10} ml^{-1}) in the stomach. In hindgut fermentors

the spore would survive transit across the stomach and come into contact with a large population of metabolically active bacteria, whereas in ruminants they would first interact with microbial populations in the rumen. Most resident GIT microbial populations would exist as mixed biofilms on the mucosal epithelium or on the surface of food particles [107]. The environment of the GIT is often exposed to low levels of antibiotics which, in some cases, has been shown to stimulate gene transfer [168,169]. In farm animals this is particularly important, since antibiotic growth promoters, such as tetracycline, have been shown to increase the probability of gene transfer. In bacteria the most common form of gene transfer is by conjugation and this can occur in *Bacillus*. In addition, efficient gene transfer can also be mediated by transduction and transformation, and *B. subtilis*, in particular, can become naturally competent. Naturally-occurring plasmids in *Bacillus* species are common and many encode conjugative or mobilisable elements. In addition, other integrated mobile genetic elements such as transposons and insertion sequences have been described [170].

The human product Enterogermina® contains a mixture of four strains of antibiotic-resistant *B. clausii* (originally reported and described as *B. subtilis*) referred to as O/C (resistance to chloramphenicol), N/R (resistant to novobiocin and rifampicin), T (resistance to tetracycline) and SIN (resistance to streptomycin and neomycin). Each of these strains was made by single and multi-step methods from a *B. clausii* strain (ATCC 9799) resistant to erythromycin, lincomycin, cephalosporins and cycloserines [8,9]. The attractiveness of a multi-resistant probiotic preparation is as an adjunct to antibiotic therapy. These resistance markers were thought to be produced by spontaneous chromosomal mutations and were not acquired. Resistance to tetracycline, rifampicin and streptomycin was stable for about 200 generations but stability to chloramphenicol was easily lost in the absence of a selective pressure [10]. One concern raised was how chromosomal mutations could provide such levels of stability in the absence of a selective pressure since chromosomal mutations that facilitate resistance are normally deleterious to cell growth and viability. Preliminary, yet inconclusive studies appear to show that the O/C, N/R, T and SIN markers are not readily transferable [10]. A recent report has characterized the erythromycin marker of the *B. clausii* strains as a new macrolide resistant gene *erm* (34) [171]. This was shown to be chromosomal and could not be transferred to *Enterococcus faecalis*, *Enterococcus faecium* or *B. subtilis* strains. Other studies have shown that of the 21 known *erm* genes some are plasmid-borne and can be transferred, these include *ermJ* in *B. anthracis* [172] and *ermC* in *B. subtilis* [173].

B. cereus (as well as *B. thuringiensis*) strains produce a broad-spectrum β -lactamase and so are resistant to pen-

icillin, ampicillin and cephalosporins. This was illustrated in a detailed characterization of the resistance profiles of 5 commercial *Bacillus* probiotics [12]. This work showed high levels of resistance to penicillin and ampicillin in two *B. cereus* products (Biosubtyl 'Dalat' and Subtyl). A third *B. cereus* product (Bactisubtil®) was shown to exhibit high levels of resistance to chloramphenicol and tetracycline. Interestingly, a plasmid from *B. cereus* carrying a tetracycline resistance gene has been transferred to a strain of *B. subtilis* and could be stably maintained [174]. Alarming, a clinical isolate of *B. circulans* has been shown to have resistance to vancomycin [175]. A strain of *Paenibacillus popillae* (formerly *Bacillus popillae*) originally dating to the 1940s has been shown to carry vancomycin resistance and to carry *vanA* and *vanB* homologues. Since vancomycin-resistant enterococci (VRE) were first reported in 1986 it has been proposed that the resistance genes in VRE and *P. popillae* shared a common ancestor. Alternatively, *P. popillae* may have been the precursor of the genes in VRE since *P. popillae* has been used as a biopesticide for over 50 years [176].

Probiotic products for use as animal feed supplements are subject to much higher levels of scrutiny than those intended for human use. The *B. cereus* strain contained in Esporafeed Plus® has been withdrawn for use in Europe as a feed additive because it was shown to carry the *tetB* gene which is normally transposon- or plasmid-borne [43]. Finally, the *B. licheniformis* strain in the feed additive AlCare™, was considered unsafe for feeding to pigs because of the risk of transferring resistance to erythromycin [177]. In conclusion, for safe use in humans and also in animal feeds, the antimicrobial resistance profiles of each probiotic strain must be clearly defined and a strong case should be made that this resistance is not transferable. In Europe the European Commission has now issued a policy statement on the assessment of probiotic bacteria resistant to antibiotics of human and veterinary importance [167].

8.3. Virulence factors

Few studies have been made of virulence factors in *Bacillus* species other than *B. anthracis* and *B. cereus*. A recent study examined 47 clinical isolates representing 14 species of *Bacillus* by examining their ability to adhere to, invade and produce cytotoxic effects in human Hep-2 and Caco-2 cells [161]. In each case the *Bacillus* species had been isolated from infected patients. Thirty-eight of the isolates were able to produce cytotoxic effects in both epithelial cell lines. These included strains of *B. subtilis*, *B. pumilus*, *B. cereus* and *B. licheniformis*. All isolates were found to adhere to both cell lines and, with the exception of *B. coagulans*, all other species carried strains that were able to invade epithelial cells. Interestingly, for *B. cereus*, not all strains were

invasive or cytotoxic. This study also examined known enterotoxin genes associated with diarrhoea. These are normally found on *B. cereus* strains but surprisingly, they were also found in one strain of *B. subtilis*. Enterotoxin genes were also found in strains of *B. thuringiensis*, *B. circulans* and *B. sphaericus*. Again, as with effects on epithelial cell lines, some *B. cereus* strains carried no enterotoxin genes. Similar studies have shown that three commercial *B. cereus* probiotics carried enterotoxin genes, produced toxins, haemolysins and lecithinases [19]. These studies show the importance of accurate determination of virulence factors and shows that no definitive statements can be made at the species level.

8.4. Product mislabeling

Unfortunately, it has become apparent that a number of commercial probiotic preparations are poorly characterized and in some cases mislabeled [178]. The reasons for this are not clear but, in part, are probably due to the lack of stringent regulations controlling the original licensing and sale of these products. In Europe products for human use as novel foods have historically been licensed if it can be shown to be of the same species as a product currently in use. As shown already, it is clear that sufficient diversity at the species level exists to preclude any assumptions being made regarding safety. This licensing strategy can explain, in part, why there are so many *Lactobacillus* products currently available. In the case of *Bacillus* products a number of these products have been mislabeled. The product Enterogermina® is one notable example. Labelled as carrying *B. subtilis* spores it has subsequently been shown to contain up to 4 strains of *B. clausii* [12,14,30]. Other examples are three Vietnamese products, all of which were shown to contain mislabeled species [12].

Another form of mislabeling is the use of non-standard bacterial nomenclature. For example, the products Lactipan Plus, Neolactoflorene, Lacto5 and Bifilact (Table 1) are all labeled as carrying *Lactobacillus sporogenes*, yet no such species exist and it has been reclassified as *B. coagulans* [6]. In the case of Neolactoflorene the spore forming species (*B. coagulans*) has been correctly identified as *B. subtilis* [179]. Other examples of invalid species names used in commercial products are *Bacillus laterosporus*, *Bacillus polyfermenticus* and *Bacillus toyoi*, the latter being a strain of *B. cereus* (var. *toyoi*). In part, the misclassification of products can be attributed to the use of crude methods for species designation and the failure to re-examine and update the taxonomic status. This does not give confidence in the standards of GMP being used. On the other hand the implication that bacterial species are related to the more commonly-used *Lactobacillus* probiotics should be considered unethical. With advanced biochemical tests (e.g., the API testing kits) and molecular

methods (e.g., 16S rRNA typing) available today it is unacceptable to mislabel products.

8.5. Product licensing

Strict regulations under the control of the EFSA are in place in Europe to control the licensing of products for use in animal feed. Only two products are currently licensed (BioPlus 2B[®] and Toyocerin[®], see Table 1). These same EU regulations governing the licensing of probiotics in animal feed has seen the withdrawal or rejection of a number of *Bacillus* products including Paciflor[®] C10 (*B. cereus*), Neoferm BS-10 (*B. clausii*), Alcare[™] (*B. licheniformis*) and Esporafeed Plus[®] (*B. cereus*). To satisfy the EFSA that a product is safe for use in animals a Risk Assessment is made of the animal feed product by an independently appointed Scientific Committee on Animal Nutrition (SCAN). The role of SCAN is to show that the use of probiotics does not constitute a risk to human health and the bacterial strains must be shown to be safe. Risks would include one or more virulence factors (e.g., enterotoxin genes), cytotoxicity, acquired antibiotic resistance markers, etc. While this European strategy has reduced the risk to the public, it is now estimated to cost approximately 1.4 million Euros to licence a product for animal use (for a detailed analysis of the costs involved in the licensing of animal probiotics in Europe see [3]). At a time when alternatives to antibiotics are clearly needed this current situation may also discourage the development of new products.

Ironically, the situation for use of probiotics in animal feed is in stark contrast to their use in human food or as 'novel foods' [178]. A case in point is *B. cereus* strain IP5832. This strain had been used extensively in the animal feed product Paciflor[®] C10 and was rejected for use in 2002 because it had been shown to produce enterotoxins that could lead to food poisoning [18]. Incomprehensibly, the same strain is still being used for human use and is marketed as Bactisubtil[®] in at least three EU countries (Belgium, Germany and Portugal) and it has recently been confirmed that this product produces at least one enterotoxin (Hbl; [19]).

As yet no regulations that match the rigor of the EFSA are in place for human products but initial steps have been taken and outlined in a joint report issued by the Food and Agriculture Organisation of the United States (FAO) and the World Health Organisation (WHO) and available through the WHO website [180] and expanded upon elsewhere [181]. The FAO/WHO report suggests a set of guidelines for a product to be used for humans whether as a stand-alone probiotic product or as a novel food supplement. These remain guidelines only and, as yet, are not strictly enforced. These guidelines state that a product must:

- (i) Be accurately defined at the genus and species level using phenotypic and genotypic methods including biochemical testing and molecular methods (e.g., 16S rRNA analysis).
- (ii) The strain must be deposited in an international culture collection.
- (iii) The strain must be assessed in vitro to determine its safety and lack of virulence factors. This would include the ability of the bacterium to adhere to, to invade and to produce cytotoxic effects in epithelial cells. Also, the absence of known enterotoxin genes, enterotoxins, haemolysins and lecithinases.
- (iv) Determination of antimicrobial activity, antimicrobial resistance profiles, absence of acquired resistance genes and inability to transfer resistance factors.
- (v) Pre-clinical safety evaluation in animal models.
- (vi) Pre-clinical assessment in animals to demonstrate efficacy.
- (vii) Phase I (safety) human trial.
- (viii) Phase II (efficacy) and Phase III (effectiveness) trials in humans.
- (ix) Correct product labeling including genus and species, precise dose and storage conditions.

While these guidelines are commendable it seems unlikely that, if enforced, many manufacturers would consider the costs of conducting Phase I to III trials. In the USA the regulations governing the licensing of probiotic bacteria are not clear, since GRAS status has not been given by the FDA to any probiotic product to our knowledge, yet numerous products are available through the internet (see Table 1). Finally, in Europe any probiotic for over-the-counter use must be evaluated rigorously (and this may include clinical trials) and a detailed dossier submitted to the European Medicines Agency in London, UK, to obtain licensing. To date, only the Italian product, Enterogermina[®] is being produced as an over-the-counter drug with current licensing in Italy, Mexico and Peru.

9. Concluding remarks

This review has summarized the current use of *Bacillus* spores as probiotics and has attempted to provide a unifying hypothesis for how they might act. We have made the case that spores are not simply passengers in the GIT but are able to germinate and proliferate within this seemingly hostile environment. This endosymbiotic life cycle enables proliferation within a host and seems to be supported by mounting evidence that *Bacillus* species are found within the gut. Some *Bacillus* species exploit the gut for pathogenesis, but most appear to grow and replicate and are ultimately excreted in the

faeces as spores. The diet, or the balance of other gastrointestinal microflora, may affect the ability of germinated spores to remain in the GIT, but it is unlikely that the known species colonise the gut. Once excreted, spores are able to survive in the environment, but, if a suitable situation arises, they can again germinate and proliferate as saprophytes. Thus, *Bacillus* species appear to be able to adopt symbiotic relationships both externally (e.g., with plants) as well as internally with whatever organism ingests them. The spore is designed to germinate in the presence of nutrients, so if germination did not occur, failure to survive in the GIT would lead to cell death, and it seems unlikely that bacteria have not adapted to this eventuality. For probiosis this ability of spores to germinate in the GIT is key to explaining the mode of action and this is likely to include immunomodulation and secretion of antimicrobials. The long-term advantages of using spores as probiotics is that they are heat-stable and can survive transit across the stomach barrier, properties that can not be assured with other probiotic bacteria that are given in the vegetative form. Whilst the use of spores as probiotics appears to be expanding, with a growing number of products available it is equally clear that supposedly 'safe' species can not be taken for granted and every product must be evaluated on a case by case basis.

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