applied microbiology

MDPI

Review

Spore Formers as Beneficial Microbes for Humans and Animals

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Abstract: Microorganisms efficiently colonize the external and internal surfaces of the animal body establishing mutually beneficial interactions and forming site- and individual-specific microbiota. The degradation of complex polysaccharides in the animal gut, the production of useful compounds, protection against pathogenic microorganisms and contribution to the development of an efficient immune system are the main beneficial effects of a balanced microbiota. A dysbiosis, an imbalanced composition of the microbiota, has been associated with a large number of diseases from gastrointestinal or urogenital disorders to allergies, cardiovascular and autoimmune diseases and even to the onset of certain cancers. A growing body of evidence has indicated that probiotic treatments, aimed at maintaining or rebalancing the microbiota, are useful to treat/prevent those illnesses. Lactic Acid Bacteria and Bifidobacteria are the most common microbes used in probiotic preparations; however, other bacteria and yeast cells are also widely used in commercial products. Here we focus on the use of bacterial spore formers as probiotics. Spore formers have been marketed as probiotics for over 50 years and are now extensively used for the treatment of intestinal disorders and as dietary supplements in humans, as growth promoters and competitive exclusion agents in animals.

Keywords: probiotics; microbiome; gut; metagenomes; spores

Citation: Saggese, A.; Baccigalupi, L.; Ricca, E. Spore Formers as Beneficial Microbes for Humans and Animals. *Appl. Microbiol.* **2021**, *1*, 498–509. https://doi.org/10.3390/applmicrobiol1030032

Academic Editor: Filipa Silva

Received: 29 September 2021
Accepted: 23 October 2021
Published: 29 October 2021

1. Introduction

Spore formers are bacteria able to form endospores (spores), quiescent cells able to survive at conditions lethal for other cells. Spore production (sporulation) is induced by a variety of environmental conditions that impair cell growth, making the sporulation process a survival strategy. Most spore formers belong to two genera of the Firmicutes phylum: The strict anaerobic Clostridia and the aerobic/facultative anaerobic Bacilli [1]. Recently, a variety of bacteria belonging to other poorly characterized genera of the Firmicutes phylum [2] have been shown to either form spore-like structures [3] or contain sporulation-specific genes [4,5], thus widening the spore formers group.

The sporulation process and the spore structure have been studied in detail in *Bacillus subtilis*, the model system for spore formers. These bacteria grow by binary fission as long as water and nutrients are available and the environmental conditions allow cell growth (vegetative cycle, Figure 1). When growth is no longer possible, cells enter the sporulation cycle, a microbial example of differentiation: (i) Asymmetric cell division occurs creating two cells of different sizes, a small prespore and a large mother cell; (ii) the mother cell engulfs the prespore, which becomes a protoplast in the mother cell cytoplasm; (iii) both cells contribute to the maturation of the prespore into the mature spore by progressive dehydration of the prespore cytoplasm and by forming a series of protective layers; (iv) the mature spore is released in the environment by the lysis of the mother cell (sporulation cycle, Figure 1). In the environment, the quiescent spore can survive for an extremely long time in the absence of water and nutrients and is resistant to the presence of UV irradiation, toxic chemicals and lytic enzymes and extreme temperatures and pH conditions [6]. The spore, however, continuously senses the environment and responds to the presence of water and nutrients, germinating and creating a new cell able to grow (Figure 1) [7].

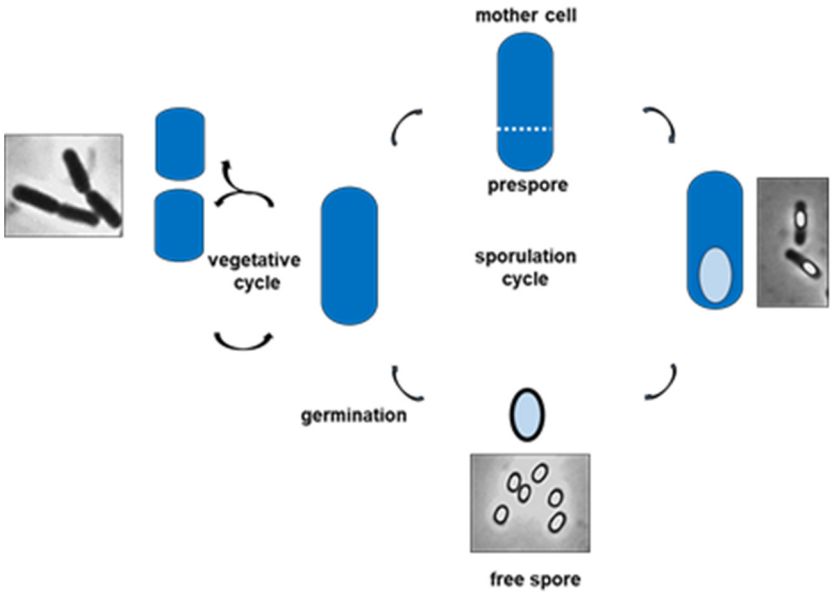

Figure 1. Vegetative and sporulation cycles of spore formers. The vegetative cycle occurs via binary fission: A cell divides symmetrically creating two identical daughter cells that grow and divide again. The sporulation cycle starts with asymmetrical cell division that creates a large mother cell and a small prespore. Then the prespore becomes a protoplast included in the mother cell cytoplasm. The formation of a series of protective layers (cortex, coat, exosporium or crust) leads to a mature spore and its release in the environment. The spore can germinate, creating a new vegetative cell able to grow and eventually sporulate again.

Survival of the spore in harsh conditions is due to the structure of the mature spore: a core, the innermost part of the spore, contains a dehydrated cytoplasm with proteins, stable RNAs and DNA and is surrounded by the cortex, a thick peptidoglycan-like layer, and by the coat, a proteinaceous multi-layered structure (Figure 2). In some species, the coat is surrounded by a further structure, the exosporium, which has an irregular shape, is loosely attached to the coat and is formed of glycoproteins [8]. In *B. subtilis*, which does not have an exosporium, an additional layer is present, namely the crust, made of proteins and glycoproteins and only visible by transmission electron microscopy (TEM) after a ruthenium red staining [6].

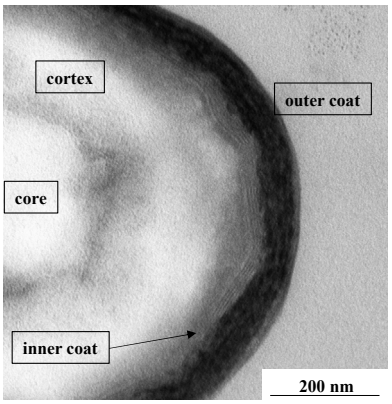

Figure 2. A *B. subtilis* spore observed by transmission electron microscopy. Core, cortex, inner and outer coat are indicated. The crust is not visible since this sample has not been stained with ruthenium red.

While anaerobic spore-formers belonging to the *Clostridium* genus are well recognized and abundant components of the animal gut microflora [9,10], members of the *Bacillus*

genus are generally considered soil bacteria. Indeed, for their exceptional longevity and resistance, *Bacillus* spores can be isolated from a variety of different environments including rocks, dust and aquatic environments [11,12]. *Bacillus* spores are also commonly isolated from the gut of various insects and animals, including humans [13,14]. In recent years, it has been suggested that *Bacillus* spores enter the animal body as food, water and air contaminants, then temporarily colonize the new habitat, becoming allochthonous gut inhabitants [14,15].

Bacillus spores have been shown to be able to perform an entire life cycle in the animal gut, entering as spores through the oral/nasal route, safely passing the gastric barrier [13,16] and germinating in the intestine [17]. In the intestine, the germination-derived cells proliferate and then re-sporulate in the colon [18,19]. The ability to perform an entire life cycle in the gut is not an exclusive property of members of the *B. subtilis* species. Other *Bacillus* species have been shown to germinate and proliferate in the gut of insects [20,21], poultry and pigs [22,23].

The common presence of *Bacillus* spores in the gastro-intestinal tract (GIT) of animals, the ability of spores and germination-derived cells to interact with intestinal cells and the ability to have beneficial effects for the animal hosts offers a rationale for the use of spore formers in probiotic products. It is, however, likely that such use was originally based on traditional foods containing spores, common in many cultures and considered to have health beneficial effects. The best-known example in this context is Natto, a Japanese traditional healthy food based on soybeans fermented with the *B. subtilis* var. natto and containing spores. *B. subtilis* var. natto produces the nattokinase, a strong fibrinolytic enzyme known to reduce systolic and diastolic blood pressure [24], whose intake may be relevant in preventing and treating hypertension [25]. Similar fermented foods are traditionally used in Korea (Chongkukjang), India (kinema), Thailand (thuo nao), Myanmar (pepok), Cambodia and Laos (sieng) and all contain spores of various *Bacillus* species, such as *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens* [26]. In addition to these species, other members of the *Bacillus* genus are currently marketed as ingredients of probiotic preparations for humans and animals. The wide commercial success of spores of *Bacillus* as probiotics or food ingredients is indubitably also due to the extreme stability of spores and their resistances to the heat treatments used for food preparation as well as long-term storage at room temperature without any loss of viability. Spore resistance overcomes problems associated with the reduction of live bacteria often encountered with probiotic preparations containing lactic acid bacteria and/or Bifidobacteria [27,28].

An overview of the use of *Bacillus* spores as probiotics is presented here, focusing on the interactions between spores and spore-derived cells with intestinal cells and their beneficial effects.

2. *Bacillus* Spores and Vegetative Cells Interact with Epithelial and Immune Cells

Both spores and cells of various *Bacillus* species interact with intestinal and immune cells through complex mechanisms still far from being entirely understood. Over the years, several reports have been published and some representative examples are summarized below.

2.1. Interaction of Bacillus Spores with Epithelial and Immune Cells

A series of in vitro and in vivo reports indicate that the interaction with spores of various *Bacillus* species modulates the immune response. Duc and collaborators [29] and Ceragioli and collaborators [30] independently analyzed the in vitro interaction of spores of *B. subtilis* with murine and human macrophages, respectively. In both cases, the efficiency of phagocytosis was modest (about 2.5%), as internalized spores rapidly germinated inside the macrophages and derived bacterial cells were quickly eliminated by both murine and human macrophages [29,30]. In an in vivo study with a murine model, orally administered spores of *B. subtilis* of a laboratory wild-type strain and an isogenic mutant unable to germinate were both able to induce similar levels of spore-specific fecal sIgA and serum

IgG, therefore demonstrating the interaction of spores with immune cells [31]. In a different study, spores of three different *Bacillus* species, *B. subtilis*, *B. licheniformis* and *B. flexus*, orally dosed to mice, promoted active lymphocyte proliferation within Peyer's patches and the production of cytokines in mesenteric lymph nodes (MLN) (IL-1α, IL-5, IL-6, IFN-γ and TNF-α) and in the spleen (IFN-γ and TNF-α) [32]. In a rabbit model, spores of *B. subtilis* and *B. anthracis* were able to bind IgM through a superantigen-like binding site and drive B cell development in the Gut-Associated Lymphoid Tissue (GALT) [33].

A more recent study showed that *B. subtilis* spores protected in vitro human keratinocytes from oxidative stress and other chemically induced injuries [34]. The same study also proposed a molecular mechanism for those effects, showing that spores adhered to the keratinocyte cell surface, inducing the nuclear translocation of the transcriptional factor Nrf-2, which in turn activated stress-response genes [34]. Antioxidant activity has also been associated with *B. megaterium* spores, both in vitro on Caco-2 cells and in vivo on a murine model of dextran sodium sulfate (DSS)-induced oxidative stress [35].

2.2. Interaction of Bacillus Vegetative Cells with Epithelial and Immune Cells

The interaction of vegetative cells of various *Bacillus* species with model intestinal cells has also been investigated. An early study showed that *B. subtilis* cells, in combination with cells of *Bacteroides fragilis*, interacted with intestinal immune cells contributing to the GALT maturation and the development of the pre-immune antibody repertoire in rabbits [36]. Interestingly, such an ability was observed with cells of a wild-type strain of *B. subtilis* and with cells of isogenic mutants impaired in general stress responses, flagellar movement or biofilm formation but not with cells of isogenic mutants unable to sporulate, suggesting that molecules produced during sporulation were essential for interaction [36]. In the same study, other spore formers such as *B. licheniformis* or *B. pumilus* were unable to affect GALT development [36]. A different in vitro study showed that vegetative cells of *B. subtilis* upregulated the expression of the Toll-like receptors TLR2 and TLR4 [32].

Fujita and co-workers [37] showed that the quorum-sensing pentapeptide ERGMT of *B. subtilis*, known as CSF (Competence and Sporulation Factor), induced synthesis of the heat-shock (HS) proteins in Caco-2 cells, which in turn prevented oxidant-induced intestinal epithelial cell injuries and loss of barrier function. CSF also showed immunomodulation and cytoprotective activities in vivo in DSS-treated mice, leading the authors to conclude that the pentapeptide has anti-inflammatory properties and that it is potentially effective for the treatment of intestinal inflammatory disorders [38]. Although CSF is a *B. subtilis*-specific molecule, not produced by other species of the genus, other *Bacillus* species, such as *B. megaterium*, *B. pumilus* and *B. clausii*, produce and secrete other molecules able to induce HS proteins [39]. The CSF-like molecules of *B. megaterium* have been characterized as a peptide smaller than 3 kDa that was also able to induce p38 MAPK phosphorylation in HT29 cells, associated with a protective response against the excess of inflammation [39]. The same strain was also able to produce a different molecule, larger than 3 kDa, responsible for strong induction of PKB/Akt phosphorylation, altogether indicating the SF185 strain of *B. megaterium* was able to alert epithelial cells against stressful conditions through secreted molecules [39].

CSF of *B. subtilis* and other molecules with a similar effect produced by various *Bacillus* species, being produced and secreted by live cells, belong to the class of post-biotics. The production of post-biotics by *Bacillus* cells is a still largely unexplored field that is likely to become the object of intense future research activities.

3. Beneficial Effects of *Bacillus* Probiotics

Although a variety of potential beneficial effects have been associated with *Bacillus* probiotics, clinical data on humans or tests on farmed animals are still limited. Most studies have been performed on animal models and have confirmed the potential of *Bacillus* spores and cells as probiotics. An example comes from an in vivo study performed with a mouse model of infection in which *B. subtilis* spores were shown to reduce the susceptibility to

the mouse pathogen *Citrobacter rodentium* [40]. *C. rodentium* is an enteric pathogen that colonizes the mouse distal colon causing epithelial lesions (crypt hyperplasia, mucosal thickening with T-cell infiltration, a highly polarized Th1 immune response and epithelial cell proliferation) similar to those caused in humans by enteropathogenic (EPEC) and enterohemorrhagic (EHEC) strains of *E. coli* [41]. Oral administration of *B. subtilis* spores one day before infection with *C. rodentium* was effective in preventing the enteropathy and drastically reducing the mortality rate in mice [40]. The reduced susceptibility to enteric pathogens caused by the ingested spores has been explained by either a "competitive exclusion" effect, with spores physically blocking the interaction between the pathogen and the intestinal cells, or the ability of spores and germination-derived cells to interact with immune cells stimulating the GALT and an adaptive immune response [40].

Many different species and strains of spore formers have been evaluated for their beneficial effects on humans and animals. In some cases, species/strains with additional beneficial properties have also been considered, such as spores of *Bacillus* strains that produce gastric-stable and strongly antioxidant carotenoids. Cells (but not spores) of a strain of *B. indicus*, producing a yellow/orange carotenoid [42], reduced plasma markers of inflammation and oxidative markers in a rat model of diet-induced metabolic syndrome, indicating that the use of carotenoid-producing strains may add additional benefits to the probiotic properties [43].

Bacillus probiotics have also been found to be effective against viral infections. In early work, heat-inactivated spores of *B. subtilis* intranasally administered to mice were able to partially protect (60% survival) the host in a challenge experiment with the influenza virus H5N2 (5 LD50) indicating the role of innate immunity and its stimulation by spores in protecting the host [44]. Killed spores were shown to stimulate TLR-mediated expression of NF-kB, control cytokine production and recruit NK cells into lungs, inducing the maturation of dendritic cells DCs [44]. More recently, it has been reported that a mixture of *B. clausii* strains is able to protect against *Rotavirus* infections in vitro [45].

Those reported above are examples of tests performed in vitro or in vivo on animal models. In the following sections, examples of beneficial effects due to *Bacillus* probiotics on farmed animals and on humans are reported. These selected examples are clearly not exhaustive of the reports present in the literature but show that a variety of species/strains have been used on a variety of hosts, analyzing a variety of different phenotypes. Therefore, in most cases, it is difficult to compare the results of the different studies. Analysis of the literature highlights the lack of a systematic approach to study the effect of *Bacillus* probiotics and underlines the need for research efforts focused on the understanding of the scientific bases of the observed effects.

3.1. Safety of Bacillus Probiotics

In Europe, a number of criteria set by the European Food Safety Authority (EFSA) must be satisfied for a *Bacillus* strain in order to be considered and commercialized as a probiotic. First, it has to belong to a list of approved QPS (Qualified Presumption of Safety) species (Table 1).

Table 1. List of approved QPS *Bacillus* species *.

Bacillus amyloliquefaciens Bacillus atrophaeus	***Bacillus megaterium***
Bacillus circulans	*Bacillus mojavensis*
Bacillus clausii	*Bacillus paralicheniformis Bacillus pumilus*
Bacillus coagulans	*Bacillus smithii*
Bacillus flexus	*Bacillus subtilis*
Bacillus fusiformis	*Bacillus vallismortis*
Bacillus lentus	*Bacillus velenzensis*
Bacillus licheniformis	

* Updated by EFSA on 7 July 2021.

In addition, each specific strain belonging to the species listed in Table 1 has to carry no significant resistance to a panel of eight antibiotics (Table 2) and exhibit no apparent toxicity in in vitro assays with cell lines (for example, HT29, Vero or Caco2 cells). A strain that meets all these requirements can be assigned the QPS status and commercialized in Europe.

Table 2. Panel of antibiotics and relative breakpoints for *Bacillus* probiotics *.

Antibiotic	Microbiological Breakpoint (mL/L)
Vancomycin	4
Gentamycin	4
Kanamycin	8
Streptomycin	8
Erythromycin	4
Clindamycin	4
Quinupristin + Dalfopristin	4

* data from The EFSA Journal (2008) 732, 1–15.

In the USA, in order for a strain to be considered as a probiotic and commercialized, it must be recognized as being Generally Recognized As Safe (GRAS), a status that can be obtained through the submission of a self-affirmed dossier to the Food and Drugs Administration (FDA).

3.2. Bacillus Probiotics for Animal Use

Many countries have strictly limited the use of antibiotics, totally banning their use as growth promoters for farmed animals. As a consequence, the search for alternatives to antibiotics has greatly increased, and probiotics, including *Bacillus* probiotics, have been identified as a potential solution [46]. The use of biological agents (probiotics) as feed ingredients is strictly regulated as indicated above, and Tables 3 and 4 report examples of spore-based products authorized to be marketed for animal use and aquaculture, respectively.

Table 3. *Bacillus* probiotics for veterinary use.

Product	Manufacturer	Species (Spores/Dose) *	Animal
AlCare	Alpharma Inc. (Australia)	*B. licheniformis NCTC 13123* (10^9–10^{10})	Swine
BioGrow	Provita Eurotech Ltd. (UK)	*B. licheniformis* [1] (1.6×10^9) and *B. subtilis* [1] (1.6×10^9)	Poultry, calves and swine
BioPlus 2B	Christian Hansen Hoersholm (Denmark)	*B. licheniformis* DSM 5749 (1.6×10^9) and *B. subtilis* DSM 5750 (1.6×10^9)	Piglets, chickens, turkeys
Esporafeed Plus	Norel, S.A. (Spain)	*B. cereus* CECT 953 (10^9)	Swine
Lactopure	Pharmed Medicare (India)	*B. coagulans* [1]	Poultry, calves and swine
Neoferm BS 10	Sanofi Sante Nutrition Animale (France)	*B. clausii* CNCM MA23/3V and CNCM MA66/4M	Poultry, calves and swine
Toyocerin	Asahi Vet S.A. (Japan)	*B. cereus var. toyoi NCIMB-40112/CNCM-1012* (>10^{10})	Calves, poultry, rabbits and swine.

[1] No information available on strain name. * Spore /dose indicated where information is available.

Examples of *Bacillus* probiotics tested in farmed animals include studies performed on chickens infected with *Escherichia coli* O78:K80, *Salmonella enterica* or *Clostridium perfringens* that were protected when pre-dosed with *B. subtilis* spores [47,48]. In a different study with chickens, diet supplementation with a mixture of *B. licheniformis* and *B. subtilis* spores resulted in increased weight gain and improved feed conversion [49]. The same mixture of *B. licheniformis* and *B. subtilis* spores was also used to feed pigs for a period of 23 weeks and was shown to reduce the incidence of diarrhea and mortality compared with a control group [50]. *B. cereus* var. toyoi, a non-toxigenic and non-pathogenic strain of *B. cereus*, reduced diarrhea and morbidity in piglets challenged with *Salmonella* [51] and improved weight gain after 6 months of treatment [52].

Table 4. *Bacillus* probiotics for aquaculture.

Product	Manufacturer	Species (Spores/Dose) *
Biostart	Microbial Solutions (South Africa); Advanced Microbial Systems (USA)	*B. megaterium* [1], *B. licheniformis* [1], *P. polymyxa* [1] and *B. subtilis* [1]
BioZyme-Aqua	Sino-Aqua Corp. (Taiwan)	*B. subtilis* Wu-S and Wu-T (10^8)
Liqualife	Cargill (USA)	*Bacillus* ssp. [1]
Promarine	Sino-Aqua Corp. (Taiwan)	*B. subtilis* [1]
Sanocare Sanolife	INVE Technologies	*Bacillus* ssp. [1]
Sanoguard	Belgium	*Bacillus* ssp. [1]

[1] No information available on strain name. * Spore/dose indicated where information is available.

Bacillus probiotics have been also used in aquaculture, as a feed supplement or directly added to the water, to improve disease resistance and increase farming productivity [53]. The supplementation of spores during egg hatching and the first stages of larvae development influenced the microbial gut composition allowing for manipulation of the microbiota. A strain of *B. subtilis* was shown to improve the survival of white shrimp, *Litopenaeus vannamei*, larvae [54] and to increase weight gain in a dose-dependent manner and colonize *Epinephelus coioides*, protecting it against bacterial (*Streptococcus* sp.) and viral (*Iridovirus*) infections [55]. A strain of *B. amyloliquefaciens* protected *Catla catla* in a challenge experiment with the pathogen *Edwardsiella tarda*, improving systemic and mucosal immunological parameters [56].

3.3. *Bacillus* Probiotics for Human Use

Probiotics for human use are obviously strictly regulated by international agencies as indicated in Section 3.1, and Table 5 reports examples of spore-based products authorized to be marketed for human use.

Table 5. *Bacillus* probiotics for human use.

Product	Manufacturer	Species (Spores/Dose) *
Bactisubtil	Marion Merrell (France); Casella-Med (Germany)	*B. cereus* ATCC 14893 (10^9)
Bibactyl	UPHACE (Vietnam)	*B. subtilis* var. natto (10^7–10^8)[9])
Bidisubtilis	Bidiphar (Vietnam)	*B. cereus* [1] (10^6)[9])
Bio-Acimin	Viet-Duc Pharm. (Vietnam)	*B. cereus* [1] and other bacteria
Bio-Kult	Probiotics international Ltd. (UK)	*B. subtilis* [1] and other bacteria
Biobaby	Ildong Pharma (Korea)	*B. subtilis* [1] (3×10^6), *C. butyricum* [1] (10^7), *B. coagulans*[1] (5×10^7)
Biosubtyl	Biophar Company (Vietnam)	*B. cereus* [1] (10^6–10^7)
Biosubtyl DL	IVAC (Vietnam)	*B. subtilis* [1] (10^7–10^8) and other bacteria
Biosubtyl I and II	Biophar Company (Vietnam)	*B. pumilus* [1] (10^6–10^7)
Biosporin	Biofarm (Ukraine)/Garars (Russia)	*B. subtilis* 2335 and *B. licheniformis* 2336 (ratio is 3:1)
Biovicerin	Geyer Medicamentos S.A. (Brazil)	*B. cereus* GM (10^6)
Bispan	Binex Co. (Korea)	*B. polyfermenticus* SCD (1.7×10^7)
Domuvar	BioProgress SpA (Italy)	*B. clausii* [1] (10^9)
Enterogermina	Sanofi Winthrop SpA (Italy)	*B. clausii* [1] (10^6)
Flora-Balance	Flora-Balance (USA)	*B. laterosporus* BOD (>10^6)
Flora3	USA	*S. boulardii* [1] and *B. coagulans* [1]
GanedenBC30	USA	*B. coagulans* GBI-30

Table 5. *Cont.*

Product	Manufacturer	Species (Spores/Dose) *
Ildong Biovita	Ildong Pharma (Korea)	*B. subtilis* [1] (3×10^6), *C.butyricum* [1] (10^7), *L. sporogenes* [1] (5×10^7)
Just Thrive	USA	*B. indicus* HU36, *B. coagulans* [1], *B. clausii* [1], *B. subtilis* HU58
Lacbon, Lacris	Uni- Sankyo (Japan)	*B. coagulans* [1]
Lactipan Plus	Istituto Biochimico Italiano SpA (Italy)	*B. subtilis* [1] (2×10^9)
Lactospore	Sabinsa Corp. (USA)	*B. coagulans* [1] (10^9)
Latero-Flora	GHC (USA)	*B. laterosporus* BOD (>10^6)
LifeinU	LHC Lesaffre Human Care Ltd. (France)	*B. subtilis* CU1
Medilac-Vita	Hanmi Pharmaceutical Co. (China)	*B. subtilis RO179* (10^8) and other bacteria
MegaSporeBiotic	UK	*B. indicus* [1], *B. subtilis* [1], *B. coagulans* [1]
Nature's First Food	Nature's First Law (California)	*B. laterosporus* [1], *B. polymyxa* [1], *B. subtilis* [1], *B. pumilus* [1]
Neolactoflorene	Newpharma S.r.l. (Italy)	*B. coagulans* [1] and other bacteria
NutriCommit	USA	*B. subtilis* [1] and *B. coagulans* [1]
Pastylbio	Pasteur Institute (Vietnam)	*B. subtilis* [1] (10^8)
Primal Defense	Garden of Life (USA)	*B. subtilis* [1] (10^8) and other bacteria
Subtyl	Mekophar (Vietnam)	*B. cereus* vietnami (10^6–10^7)
SunnyGreen Cleansing	USA	*B. coagulans* [1]
Sustenex	Ganeden Biotech Inc. (USA)	*B. coagulans Ganeden BC30*
THORNE	USA	*B. coagulans* [1]

[1] No information available on strain name. * Spore/dose indicated where information is available.

Furthermore, in this case, a large number of reports are present in the literature, but in this review, we will limit the discussion to a few, select examples.

Piewngam and collaborators [57], upon screening human populations from rural and urbanized areas, observed an inverse correlation between the presence of *Bacillus* species (mainly *B. subtilis*) and the absence of the pathogen *Staphylococcus aureus*. In particular, *S. aureus* was never detected in fecal samples of adults when *Bacillus* species were present. Interestingly, such pathogen exclusion was not limited to the site of interaction (the gut) but was also observed in other colonization sites. Indeed, *S. aureus* never colonized the nasal mucosa in the presence of intestinal *Bacillus*, even if *Bacillus* was not present in the nose [57]. In the same study, the authors showed that the cyclic lipopeptide fengycin, produced by most *B. subtilis* strains, competed with the AIP signal molecule of the Agr quorum-sensing system of *S. aureus*, essential for the virulence of the pathogen [57]. Moreover, the oral administration of a *B. subtilis* strain, but not an isogenic mutant not producing fengycin, completely blocked *S. aureus* colonization in mice, elegantly demonstrating the mechanism of action of *B. subtilis* protection against *S. aureus* infections [57].

Another example comes from a recent study in which a carotenoid-producing strain of *B. indicus* was shown to deliver its carotenoid to the human body causing an increased systemic carotenoid accumulation and improving the barrier functions [58]. In this study, over 60 healthy overweight or obese volunteers showed increased concentrations of bacterial carotenoid in plasma samples after three and six weeks of daily supplementation of the carotenoid-producing probiotic [58].

4. Future Development

Although the use of bacterial spores in commercial probiotic products is already common practice (Tables 3–5), new studies addressing the health beneficial effects of *Bacillus* probiotics, focused on the isolation of novel strains and on the mechanisms by which they exert their beneficial effects on humans and animals, are continuously arising.

Some of these studies are opening new fields in the *Bacillus* probiotic world, proposing the *Bacillus* spore as a platform to deliver beneficial molecules, thus combining the probiotic and additive beneficial effects. Other studies have highlighted that *Bacillus* probiotics may have beneficial effects on neurodegenerative diseases, opening the possible use of probiotics for new therapeutic targets. Examples of such applications and new therapeutic targets are summarized below.

4.1. Functional Spores as Probiotics

A strategy has recently been developed to functionalize spores by adsorbing active molecules on their surface [59]. The strategy has been initially developed using a laboratory collection strain of *B. subtilis* and later tested on a probiotic strain of *B. subtilis* [60] and *B. megaterium* [61]. This strategy allowed a non-recombinant display of enzymes that are stabilized by the interaction with the spore and keep their activity at conditions that also inactivate the free enzymes [59,61]. Spore adsorption is based on the spontaneous binding of molecules to the spore surface without the need for chemical treatment (crosslink) and has been recently reviewed [62]. Being a non-recombinant strategy, spore adsorption allows the functionalization of spores of probiotic strains, adding new beneficial effects, due to the adsorbed molecule, without posing any safety issues. A simple example could be the adsorption of enzymes for the degradation of detrimental molecules, such as food components that induce allergies or intolerances in specific groups of people. However, functional spores have not been tested yet on animal models and their development is still in progress [62].

4.2. Bacillus Probiotics and the Nervous System

Two recent papers have shown that *Bacillus* probiotics may have beneficial effects on neurodegenerative diseases [63,64], opening a new frontier to the beneficial effects associated with *Bacillus* probiotics. Goya et al. showed that both spores and cells of various *B. subtilis* strains, through secreted molecules and biofilm formation, inhibit α-synuclein aggregation and clear preformed aggregates [64]. Cogliati et al. identified the Competence and Sporulation Factor (CSF) of *B. subtilis*, together with biofilm formation, as involved in the anti-Alzheimer's Disease (AD) effects observed in a *C. elegans* model of AD [63]. In such a model, *B. subtilis* delayed age-related neurodegeneration and cognitive damage and alleviated paralysis defects and behavioral deficits, all induced in transgenic worms by the expression of the Aβ peptide [63]. These two studies, although performed on a *C. elegans* model, offer exciting possibilities for probiotic treatments and the development of drug therapies based on *Bacillus* molecules to target neurodegenerative diseases.

5. Conclusions

This review summarizes select examples of the interaction of spores and/or cells of various *Bacillus* species with epithelial and immune cells, and the beneficial effects exerted by *Bacillus* probiotics in in vitro and in vivo model animals, in farmed animals and in humans. Rather than being exhaustive of what is present in the literature, these selected examples aim to provide an overview of what is known about various *Bacillus* species/strains, various hosts and various phenotypes, underlining the need for a more systematic approach to study *Bacillus* probiotics. Focusing on a single species/strain, a single host and looking at selected phenotypes would it make possible to compare results and address the molecular basis of the effects asserted.

Particularly promising is the possibility of using spores of probiotic strains to deliver health-beneficial molecules to the intestinal or nasal mucosa. Such non-recombinant

delivery would combine the beneficial effects of the probiotic to those of the added molecule, with the possibility to deliver active enzymes to degrade food components with allergenic potentials or anti-inflammatory or antioxidant properties. Although these functionalized spore probiotics have not been tested yet in vivo, spores displaying antigens have long been used as oral and nasal vaccines able to induce protective antigen-specific immune responses [62], therefore providing a solid base for their safety and beneficial potentials.

Author Contributions: A.S., L.B. and E.R. wrote, edited and revised the manuscript draft. All authors have read and agreed to the published version. All authors have read and agreed to the published version of the manuscript.

Funding: No specific funding was received to support this work.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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