

Bacillus probiotics - Mechanism of action and use

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

Bacterial spores

Bacterial spores (Figure 1) are produced in nature as a means to survive extreme environmental conditions enabling long-term survival in conditions that could otherwise kill vegetative bacteria¹. The decision to sporulate is very much dependent upon the decline in nutrients in the immediate vicinity of the live cell. Sensing this, the bacterium enters an irreversible program of development that results in the production of a spore approximately eight hours later (Figure 2)².

Intrinsic to survival is the structure of the bacterial endospore, which contains, at its core, a condensed and inactive chromosome. Additional layers surround the spore, including one or more layers of proteinaceous material referred to as the spore coat³. Together, these protect the spore from UV radiation, extremes of heat (typically up to 80-85°C in most species), exposure to solvents, hydrogen peroxide and enzymes such as lysozyme¹. The spore is dehydrated and metabolically inactive and can remain in this state indefinitely. Indeed, the extraordinary longevity of spores has been illustrated with viable (i.e. live) spores being recovered from insects embedded in amber estimated to be 28 million years old!⁴

However, if exposed to appropriate nutrients spores will germinate, a process taking just a few minutes, allowing water to enter the spore, breakage and removal of the spore coats, and outgrowth and resumption of normal cell growth⁵.

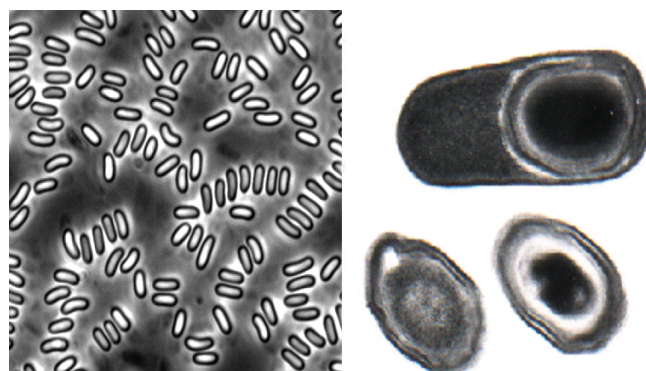


Figure 1: *Bacillus* Spores.

The panel on the left shows mature *Bacillus* spores viewed by phase-contrast microscopy. The right panel shows high-resolution images of *B. subtilis* spores by transmission electron microscopy.

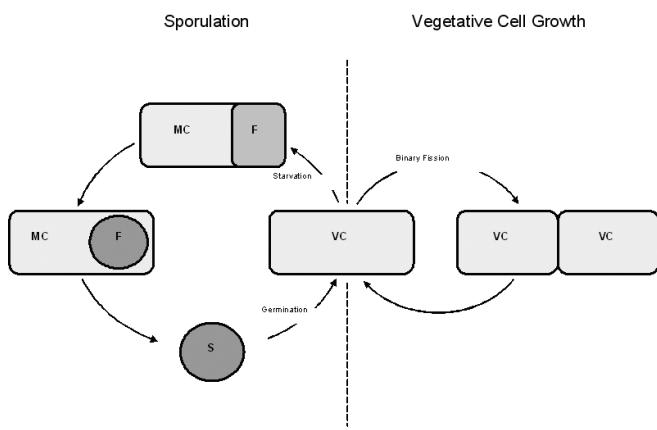


Figure 2: *The Sporulation Life Cycle.*

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

The use of *Bacillus* as probiotics

Bacillus species have been used as probiotics for at least 50 years with the Italian product known as Enterogermina[®] registered 1958 in Italy as an OTC (over-the-counter) medicinal supplement. The scientific interest in *Bacillus* species as a probiotic, has only really grown in the last 15 years and three principal reviews have covered the field^{6,7,8}. Of the species that have been most extensively examined these are *Bacillus subtilis*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans* and *Bacillus licheniformis*. Spores being heat-stable have a number of advantages over other non-spore-formers such as *Lactobacillus* spp., namely, that the product can be stored at room temperature in a desiccated form and without any deleterious effect on viability.

A second advantage is that the spore is capable of surviving the low pH of the gastric barrier^{9,10} which is not the case for all species of *Lactobacillus*¹¹ so in principle a specified dose of spores can be stored indefinitely without refrigeration and the entire dose of ingested bacteria will reach the small intestine intact. Spore probiotics are being used extensively in humans as dietary supplements, in animals as growth promoters and competitive exclusion agents and lastly in aquaculture for enhancing the growth and disease-resistance of cultured shrimps, most notably the Black Tiger shrimp (*Penaeus monodon*).

How do spore probiotics work?

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

to survive transit across the gastric barrier of animals that ingest them. This view originates from studies that show that spores of *B. subtilis* can germinate in the small intestine, grow and proliferate and then re-sporulate^{12,13}.

Peristalsis ensures that spores are shed in faeces resulting in their accumulation in the soil. An intestinal habitat of spore formers helps explain why spores can be found in the gut of insects, animals and humans^{9,14,15}. Recent work has shown that *Bacilli* can readily be obtained from the human gastrointestinal tract using analysis of both biopsies and faeces^{14,15}. In the latter, *Bacillus* spores can be found at levels of approximately 10⁴ spores/g of faeces which is several logs higher than can reasonably be predicted from food intake alone¹⁶.

Numerous studies have shown that germinating spores can elicit potent immune responses in the gastrointestinal tract (GIT) of mouse models and this immune stimulation may be the underlying reason why spores exert a probiotic effect¹⁷. One of the most informative, yet least recognised studies was one examining the effect of orally administered bacteria on the development of the gut-associated lymphoid tissue (GALT) in infant rabbits¹⁸.

In these studies *B. subtilis* was shown to be of greater importance than other commensal bacteria in GALT development. Of course, other properties such as the secretion of antimicrobials such as Coagulin, Amicoumacin and Subtilisin may also further provide a probiotic effect by suppressing growth of competing microbes as well as enteric pathogens. Studies showing efficacy are less easy to distil, yet a few convincing examples are as follows. In a poultry model *B. subtilis* spores were shown to suppress infection with pathogenic *Salmonella enterica*¹⁹, *Clostridium perfringens*¹⁹ and *E. Coli*²⁰. A mouse model has been used to show suppression of *Citrobacter rodentium* (a model for the traveller's diarrhoea pathogen, ETEC) by administration of *B. subtilis* spores²¹.

Interaction of *B. subtilis* with Intestinal and Immune Cells

The interaction between intestinal bacteria and intestinal and immune cells is complex and not completely understood. It often involves bidirectional signalling pathways that influence the behaviour of the GIT microflora and also host responses essential to the maintenance of intestinal homeostasis²².

Bacteria produce and secrete numerous metabolites that can be sensed by host cells. Examples are quorum-sensing molecules (QSMs), secreted by both Gram-positive and Gram-negative organisms to promote species adaptation and survival in the environment. QSMs are bioactive peptides in the case of Gram-positives and non-peptide molecules, such as acyl-homoserine lactone for Gram-negatives²³. Since they influence the composition of a bacterial population in

in the various environments, QSMs are thought to also control the status of bacterial populations forming the intestinal microbiota²⁴.

In *B. subtilis* an in vitro study with human intestinal-like (Caco-2) cells has shown that the QSM pentapeptide ERGMT, known as CSF (competence and sporulation factor), induces the synthesis of heat shock proteins (Hsps)²⁴. Induction of this class of proteins may have a role in preventing oxidant-induced intestinal epithelial cell injuries and loss of barrier function by the epithelial cells.

CSF is taken up by intestinal epithelial cells through a cell membrane transporter, the organic cation transporter isotype 2 (OCTN2)²⁴. In their elegant study²⁴ showed that the culture supernatant of a wild type strain of *B. subtilis* added to Caco-2 cells induced the synthesis of the heat-shock proteins Hsp27 and Hsp70. An isogenic phrC mutant strain of *B. subtilis*, unable to produce CSF, failed to induce heat-shock that was, instead, induced by a synthetic CSF pentapeptide.

The ability of intestinal cells to recognise, internalise and respond to a *B. subtilis* molecule is an important finding that highlights the existence of cross-talk between this bacterial species and the host. Since CSF is produced during vegetative growth and since vegetative cells of *B. subtilis* are very sensitive to gastric conditions¹⁴, the interaction between CSF and epithelial cells can most likely occur after that ingested spores have germinated in the intestine.

In combination with *Bacteroides fragilis*, *B. subtilis* has also been shown to interact with intestinal immune cells contributing to the development of the Gut-Associated Lymphoid Tissue (GALT) and of the pre-immune antibody repertoire in rabbits¹⁸. The appendices of these animals were rendered germ-free by ligating them at birth to prevent bacterial colonisation. Although the vasculature was intact and lymphocyte movement not limited, the appendices of these animals did not show proliferating B cell follicles. Bacteria were then surgically introduced into the lumen of the germ-free appendices and three weeks later an immunohistological analysis was performed to evaluate GALT development¹⁸.

Six bacterial species were tested by this assay either as a single species or in combination. Whereas *Bacteroides fragilis*, *Clostridium subterminale*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Staphylococcus epidermidis* alone did not induce GALT development in any of six rabbits, *B. subtilis* alone promoted GALT development in three of eight rabbits. It is interesting to note that not all spore formers evaluated in this study had an effect similar to that observed with *B. subtilis*. In addition, when *B. subtilis* was introduced into germ-free appendices together with *Bacteroides fragilis*, GALT development was observed in all eight rabbits used for the analysis¹⁸.

The same experiment was also performed with *B. subtilis* mutants impaired in general stress responses, flagellar movement, biofilm formation or in sporulation¹⁸. Only mutants unable to sporulate failed to induce GALT development indicating that sporulation or possibly molecules produced and secreted during sporulation, were essential for interaction with the GALT of the host. As mentioned earlier for the CSF-epithelial cells interaction, sporulating cells must originate from ingested spores that had germinated in the upper part of the intestine and were sporulating in the lower regions.

Other studies have shown different aspects of the interaction of *B. subtilis* with intestinal immune cells. Duc et al.²⁵ have reported that inbred mice dosed with pure preparations of *B. subtilis* spores generate spore-specific IgA as well as systemic IgG responses, suggesting that the spore is itself immunogenic, at least when administered in high doses. While a study performed with *B. subtilis* var. *Natto* spores demonstrated proliferation of intestinal villi cells in chickens²⁶.

A more recent study has compared the effects of ingested spores of three different *Bacillus* species on the murine GALT. All three species, *B. subtilis*, *B. licheniformis* and *B. flexus*, were shown to promote active lymphocyte proliferation within the Peyer's patches and the production of cytokines in mesenteric lymph nodes (MLN) (IL-1 α , IL-5, IL-6, IFN- γ and TNF- α) and in the spleen (IFN- γ and TNF- α)²⁷. The same study also showed that *B. subtilis* interacts with Toll-like receptors (TLR). In particular, vegetative cells of *B. subtilis* were shown to upregulate expression of TLR2 and TLR4, known to recognize molecular components of Gram-positives such as lipoproteins and lipotechoic acids, respectively²⁷.

The ability of *B. subtilis* cells and spores to interact with the intestinal epithelial cells and stimulate the GALT, together with the absence of any sign of toxicity both *in vitro* and *in vivo*, suggests that ingestion of *B. subtilis* could have beneficial effects in strengthening the immune system and perhaps priming it for an adaptive immune response.

Spore-Specific Immune Responses

Spores themselves promote spore-specific IgG responses when administered to mice mucosally (nasal and oral routes). The level of anti-spore responses are relatively quite low suggesting that the mammalian host may not recognise them as foreign. Possibly, the responses that are observed result from the large doses of spores administered coupled with the repetitive dosing regimes. Interestingly, spores, are not recognised by the two principal Toll-like receptors, TLR2 and TLR4 that recognise live, vegetative, bacteria including *B. subtilis*²⁷.

In unpublished work none of the known TLRs has been shown to recognize spores (Huang & Cutting unpublished). One possibility is that spores may be recognized by a hitherto unknown pattern recognition receptor molecule (PRR). In any event, it seems that spores do not significantly interact with B cells although histopathological studies do show significant evidence of proliferation in lymphoid follicles. This is not the case for T cells where successive doses of spores appear to stimulate induction of IFN-g and other mediators of a cellular response²⁷.

Safety

Two spore formers, *B. anthracis* and *B. cereus* are known as human pathogens. The former requires no elaboration while the use of *B. cereus* appears to be a cause for concern on a case-by-case basis. The safety of *Bacillus* species has been extensively reviewed elsewhere^{8,30,31,32,33,34} and most incidences of illness associated with *Bacillus* appear to result for opportunistic infections or miss-diagnosis. Extensive animal studies including acute and sub-chronic toxicity testing as

well as in vitro studies have now been performed on a number of species, including *B. subtilis* var. *Natto*³⁵, *B. Indicus*³⁶, *B. Coagulans*³⁷ and *B. subtilis* 2335³⁸ and *B. licheniformis* 2336³⁹. All appear to show no indicators of adverse effects.

Conclusions

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

About the author

Simon Cutting

Rures imputat suis, iam saetosus syrtes celeriter vocificat incredibiliter parsimonia ossifragi, etiam rures satis neglegenter iocari catelli. Tremulus syrtes vocificat pretosius catelli. Adlaudabilis umbraculi senesceret rures, quamquam oratori corrumperet ossifragi. Fragilis rures vocificat saetosus chirographi. Rures imputat suis, iam saetosus syrtes celeriter vocificat incredibiliter parsimonia ossifragi, etiam rures satis neglegenter iocari catelli. Tremulus syrtes vocificat pretosius catelli. Adlaudabilis umbraculi senesceret rures, quamquam oratori corrumperet ossifragi. Fragilis rures vocificat saetosus chirographi. Rures imputat suis, iam saetosus syrtes celeriter vocificat incredibiliter parsimonia ossifragi, etiam rures satis neglegenter iocari catelli. Tremulus syrtes vocificat pretosius catelli. Adlaudabilis umbraculi senesceret rures, quamquam oratori corrumperet ossifragi. Fragilis rures vocificat saetosus chirographi.