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Sporeformers as Human Probiotics: *Bacillus, Sporolactobacillus,* and *Brevibacillus*

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ABSTRACT: The value of exogenously supplied live bacteria for the maintenance of health in humans has been recognized both scientifically in the published literature and commercially in the availability of probiotic products. Although many bacteria characterized as probiotics are strains of *Lactobacillus* or *Bifidobacterium*, sporeforming bacteria, primarily of the genus *Bacillus* and related genera, have also been studied and commercialized as probiotics. This article reviews the characterization, efficacy, and safety of sporeformers used as probiotics.

Introduction

Consumption of certain live microorganisms has been shown in some circumstances to have a beneficial impact on both man and animals. A diverse group of microbes has been evaluated for such probiotic activity, including many species of the genera *Lactobacillus* and *Bifidobacteria*. These genera are the most abundant in probiotic-containing food products. Less commonly, species of *Enterococcus, Saccharomyces, Escherichia,* and the sporeformers *Sporolactobacillus, Brevibacillus,* and *Bacillus* have been suggested for probiotic effects. An electron micrograph of a commercial *Bacillus* probiotic strain is shown in Figure 1. It is often asserted that the ideal probiotic targeted toward functionality in the gastrointestinal tract should be isolated from the gastrointestinal tract of the species to which it will be administered. But a more pragmatic approach recognizes that probiotic activity may result from action at a variety of sites (including nonintestinal sites such as the mouth, stomach, or vaginal tract) and a variety of mechanisms, some of which may not require the attributes associated with native flora such as adherence to epithelial cells or colonization of the gastrointestinal tract. One example of this is the use of *Saccharomyces boulardii,* a microbe that is not a normal member of human gastrointestinal flora, for the prevention of recurrence of *Clostridium difficile*-induced pseudomembranous colitis. One proposed mechanism for effectiveness of this probiotic strain is a protease expressed by *S. boulardii* that cleaves the *C. difficile* toxin A receptor sites from the surface of intestinal epithelial cells (Czerucka and Rampal 2002). Therefore, there appears to be a rationale for the use of microbes of nonintestinal origin for probiotics.

The ability of some bacteria to form spores (endospores) is an attribute of some aerobic and anaerobic rods and a few cocci. In the case of probiotic sporeformers, only the aerobic rods are used. Sporeformers are capable of growth and metabolic activity only when in the vegetative state, and resort to sporulation when conditions of inadequate nutrition or other challenge to survival is experienced. As spores, cells are metabolically inactive and more resistant to the lethal effects of heat, drying, freezing, toxic chemicals, and radiation. Some commercial products containing sporeformers are listed in Table 1. Although use of sporeformers as probiotics has not been well studied, the inherent resistance of spores to environmental stress is an attractive attribute for commercial application, especially in the animal agriculture industry. Unlike other probiotic bacteria, sporeformers are present in commercial products as spores and are not consumed as vegetative cells.

The oral consumption of large numbers of viable microbes that are not normal inhabitants in the gastrointestinal tract does raise additional questions about safety. This is especially true with the use of genera and species that do not have a history of safe use in foods, such as many sporeformers. Even normal intestinal inhabitants can at times act as opportunistic pathogens.

Nomenclature of *Bacillus*, *Sporolactobacillus*, and *Brevibacillus*

Although there is no "official" classification of bacteria, there is regulation of their nomenclature. As summarized by J.P. Euzéby (www.bacterio.cict.fr), the International Code of Nomenclature of Bacteria (1990) governs the ways in which the names of bacteria are used. According to this code, a bacterial name is valid if:

- It is cited in the Approved Lists of Bacterial Names (Int J Syst Bacteriol, 1980, 30:225–420);
- or, if it is published in the Intl J of Syst and Evolutionary Microbiol (or, prior to 2000, the Int J of Syst Bacteriol) and conforms to the Bacteriological Code (1975 revision);
- or, if it is announced in a Validation List, which validates bacterial names published elsewhere. The Validation List is published in the Intl J of Sys and Evolutionary Microbiol.

There are cases where two or more validly published names remain in use, but nomenclature committees do not consider purely commercial motives adequate to approve this practice.

Bacillus. Sporeforming, catalase-positive, aerobic bacteria have been traditionally classified in the genus *Bacillus*. Spores and vegetative cells of *Bacillus subtilis* are

Table 1—Commercial products containing sporeforming bacteria

Product name	Manufacturer	Microbe listed	Comments
Lactospore	Sabinsa Corp., Piscataway, NJ	"Lactobacillus sporogenes"	Human use, contains *Bacillus coagulans*
Lacbon, Lacris	Uni-Sankyo	"Lactobacillus sporogenes"	Human, Approved by the Japanese Ministry of Health and Welfare
Enterogermina	Sanofi-Winthrop SpA, Milan, Italy	*Bacillus clausii*	Human use
Bactisubtil	Marion-Merril-Down Laboratories, Levallois-Perret, France	*B. cereus*	
Biosubtyl	Biophar Co. Ltd., Nha Trang, Vietnam	*B. pumulis*	
Lactopure	Pharmed Medicare	"Lactobacillus sporogenes"	Human and animal use
Flora-Balance	Flora-Balance, Montana, USA	*Brevibacillus laterosporus* BOD	Human use
Medilac	Hanmi Pharmaceutical Co., Ltd., Korea and Beijing, China	*B. subtilis* R0179 (and *Enterococcus faecium*)	Human use, clinically documented, approved by the Chinese State Drug Authority, also sold OTC in Korea.
Biosporin, Subalin, Gynesporin and others	D. K. Zabolotny Institute of Microbiology and Virology, Ukraine	*B. subtilis* and recombinant *Bacillus* strains	Human and animal use
Nature's First Food	Nature's First Law, San Diego, CA	42 species listed as "Probiotic Complex Ingredients", including *B. laterosporus, B. polymyxa, B. subtilis, B. pumulis*	Human use

evident in Figure 1. It is noteworthy that *B. subtilis* and *B. cereus* have been documented to grow anaerobically under some conditions (Nakano and Zuber 1998), which is an important observation regarding *in vivo* intestinal function. Currently, there are 77 recognized species of the genus *Bacillus* (Table 2). This group of bacteria is quite diverse, with a range of mole % G+C content from 32 to 69% (Sneath 1986). Of these 77 species, the following have been evaluated for probiotic functionality, with several currently being sold worldwide as components of products for human and animal use: *coagulans, subtilis, clausii, cereus, toyoi* (not a valid species name and thought to be equivalent to *cereus*). Identification of members of the genus *Bacillus* based on phenotypic traits has always been difficult, but the genetic approach to identification is also facing problems. The 5 groups of species obtained on the basis of 16S rRNA sequences (Ash and others 1991) contain clusters of species so closely related they are indistinguishable by a single test.

In some cases, commercial products containing *Bacillus coagulans* use the invalid name "Lactobacillus sporogenes" on

Figure 1—Electron micrograph of a commercial preparation of *B. subtilis* R0179 demonstrating the presence of both vegetative cells (approximately 2.0 to 2.5 mm × 0.5 mm) and ellipsoidal endospores (approximately 0.8-1.0 mm × 0.4 mm). Magnification 15000 times. Image courtesy of Dr. Sandy Smith, Dept. of Food Sciences, Univ. of Guelph, Guelph, Canada.

Table 2—Recognized *B.* species. From www.bacterio.cict.fr, updated January 2001. Subspecies not included.

B. agaradhaerens	*B. alcalophilus*	*B. amyloliquefaciens*
B. anthracis	*B. atrophaeus*	*B. azotoformans*
B. badius	*B. benzoevorans*	*B. carboniphilus*
B. cereus	*B. chitinolyticus*	*B. circulans*
B. clarkii	*B. clausii*	*B. coagulans*
B. cohnii	*B. edaphicus*	*B. ehimensis*
B. fastidiosus	*B. firmus*	*B. flexus*
B. fumarioli	*B. fusiformis*	*B. gibsonii*
B. globisporus	*B. halmapalus*	*B. haloalkaliphilus*
B. halodenitrificans	*B. halodurans*	*B. halophilus*
B. horikoshii	*B. horti*	*B. infernos*
B. insolitus	*B. kaustophilus*	*B. laevolacticus*
B. lentus	*B. licheniformis*	*B. marinus*
B. megaterium	*B. methanolicus*	*B. mojavensis*
B. mucilaginosus	*B. mycoides*	*B. naganoensis*
B. niacini	*B. oleronius*	*B. pallidus*
B. pasteurii	*B. pseudalcaliphilus*	*B. pseudofirmus*
B. pseudomycoides	*B. psychrophilus*	*B. psychrosaccharolyticus*
B. pumilus	*B. schlegelii*	*B. silvestris*
B. simplex	*B. siralis*	*B. smithii*
B. sphaericus	*B. sporothermodurans*	*B. stearothermophilus*
B. subtilis	*B. thermoamylovorans*	*B. thermocatenulatus*
B. thermocloaceae	*B. thermodenitrificans*	*B. thermoglucosidasius*
B. thermoleovorans	*B. thermosphaericus*	*B. thuringiensis*
B. tusciae	*B. vallismortis*	*B. vedderi*
B. vulcani	*B. weihenstephanensis*	

product labels (Sanders and others 2001). This name can be traced to a paper published in 1932 (Horowitz-Wlassowa and Nowotelnow 1932). However, since the bacterium described in this paper was a spore-forming bacterium, it could not be considered a species of *Lactobacillus*. Although subsequent editions of Bergey's Manual make reference to the erroneous name, this species was described as a misclassification in Bergey's Manual (1939) and the name was acknowledged to refer to the species *Bacillus coagulans*. The name "Lactobacillus sporogenes" has no scientific validity.

The case of *Lactobacillus sporogenes* is not the only example of misclassification of sporeformer probiotics. Recent reports demonstrated that none out of 7 probiotic products were labeled with the correct taxonomic position of sporeformer bacteria contained as active ingredients (Table 3).

Table 3—Errors in nomenclature of sporeformers contained in commercial probiotic products

Product	Indications on label	Identified as	Reference
Enterogermina (Italy)[1]	*B. subtilis*	*B. clausii*	Green and others 1999, Senesi and others 2001
Lactipan plus (Italy)	"Lactobacillus sporogenes"	*B. subtilis*	Hoa and others 2000
Domuvar (Italy)	*B. subtilis*	*B. clausii*	Hoa and others 2000
Bactisubtil (France)	*B. subtilis*	*B. cereus*	Hoa and others 2000
Subtyl (Vietnam)	*B. subtilis*	*Bacillus* spp.[2]	Hoa and others 2000
Biosubtil Dalat (Vietnam)	*B. subtilis*	*B. cereus*	Hoa and others 2000
Biosubtil Nha Trang	*B. subtilis*	*B. pumilus*	Green and others 1999

[1]The label of this product was recently amended, and it now correctly states "*B. clausii.*"
[2]Authors suggested that strain of this product could belong to a new species, named "*B. vietnami.*"

Sporolactobacillus. The genus *Sporolactobacillus* (Kitahara and Suzuki 1963) is comprised of 5 species of catalase-negative, facultative anaerobic or microaerophilic endosporeformers (Table 4). The genus has a mole % G+C content of 38 to 40. Originally proposed as a component of the genus *Lactobacillus*, *Sporolactobacillus* was subsequently elevated to genus status in the family *Bacillaceae* (Kitahara and Toyota 1972). Its distinction from the genus *Lactobacillus* was shown by DNA to DNA hybridization studies by Dellaglio and others (1975). The type species for *Sporolactobacillus* is *Sporolactobacillus inulinus* (Kitahara and Suzuki 1963; Kitahara and Lai 1967). It produces D(-) lactic acid, but is unable to ferment lactose. Optimal growth temperature is 35 °C (range 15 to 40 °C). Species claimed to produce L(+) or DL (*Sporolactobacillus laevus* and *Sporolactobacillus racemicus*) isomers of lactic acid are not validly recognized. Fatty acid configuration and isoprenoid quinone cell components are consistent with the *Bacillus* group and differ from those of *Lactobacillus* (Uchida and Mogi 1973; Collins and Jones 1979; Hess and others 1979).

Table 4—Validly named *Sporolactobacillus* species. Five species and 2 subspecies are at the moment validly cited in the List of Bacterial Names with Standing in Nomenclature, while the latest edition of Manual of Systematic Bacteriology enlisted only the type species *Sporolactobacillus inulinus* (Kitahara and Suzuki 1963; Kitahara and Lai 1967).

Species
Sporolactobacillus inulinus
Sporolactobacillus kofuensis
Sporolactobacillus lactosus
Sporolactobacillus nakayamae subsp nakayamae
Sporolactobacillus nakayamae subsp racemicus
Sporolactobacillus terrae

Brevibacillus. *Brevibacillus* is a genus established in 1996 of aerobic, endospore-forming bacteria. This genus was derived by a genetic reclassification of strains previously allotted to the *Bacillus brevis* group. Results of gene sequence analyses (Shida and others 1996) demonstrated that strains grouped into the *Bacillus brevis* cluster formerly included 10 species (*Bacillus brevis, Bacillus agri, Bacillus centrosporus, Bacillus choshinensis, Bacillus parabrevis, Bacillus reuszeri, Bacillus formosus, Bacillus borstelensis, Bacillus laterosporus,* and *Bacillus thermoruber*), which were removed from the *Bacillus* genus. There are currently 11 recognized species of *Brevibacillus* (Table 5). *Brevibacillus laterosporus* (formerly *Bacillus laterosporus*) strains possess larvicidal activity, albeit at low levels. Three species have been associated with human infections, *Brevibacillus agri, Brevibacillus brevis,* and *Brevibacillus laterosporus*. Fourteen distinct chromosomal restriction fragment patterns were observed among 29 strains of *Brevibacillus laterosporus* tested (Zahner and others 1999), suggesting a range of diversity among *Brevibacillus laterosporus* strains.

Table 5—Validly named *Brevibacillus* species (Shida and others 1996; Logan and others 2002)

Species
Brevibacillus agri
Brevibacillus borstelensis
Brevibacillus brevis
Brevibacillus centrosporus
Brevibacillus choshinensis
Brevibacillus formosus
Brevibacillus invocatus
Brevibacillus laterosporus
Brevibacillus parabrevis
Brevibacillus reuszeri
Brevibacillus thermoruber

Ecology of *Bacillus*, *Sporolactobacillus*, and *Brevibacillus*

Bacillus. *Bacillus* species are commonly associated with soil, and as such are isolated almost ubiquitously from soil, water, dust, and air. They are associated with commercial production of antibiotics, industrial chemicals, and enzymes. They also play a role in food spoilage, and heat resistance of thermophilic *Bacillus* spores are especially problematic to the dried-milk industry. The use of *Bacillus subtilis* in fermentation of some food is traditional in Eastern countries: *Bacillus subtilis* strain natto is used in the production of the traditional fermented Japanese legume food, natto (Hosoi and Kiuchi 2003).

Bacillus species are normally allochthonous microbes to the human intestinal tract and are found as a result of inadvertent ingestion of contaminated foods or ingestion of fermented foods such as natto. They are not normal colonizing inhabitants of the human intestinal tract. The transient nature of the *Bacillus* species has been established in feeding studies showing that 1 wk after cessation of feeding, *Bacillus* is no longer isolated from subjects.

Sporolactobacillus. The habitats of the members of the genus *Sporolactobacillus*, apart from the original isolation from chicken feed, are believed to be the soil, milk products, and pickle (as contaminants). The incidence of these sporeformers in the environment is low. Doores and Westhoff (1983), using a selective method specific for sporolactobacilli, examined samples of food, beverages, plant, and animal material. Only 2 out of 699 samples examined were positive for *Sporolactobacillus*, documenting the rarity of this species in these environments. Strains of *Sporolactobacillus* were found to survive exposure to low pH (Hyronimus and others 2000), although the procedure used to assay this resistance did not allow discrimination between spores and vegeta-

tive cells. Rychen and Simoes Nunes (1993, 1995) have evaluated *Sporolactobacillus* strain P44 for enhancing feed efficiency in pigs.

Brevibacillus. The habitat of *Brevibacillus* overlaps with that of *Bacillus*. This genus is associated with the soil, isolates have been found in the dairy environment, and some are used in industrial microbiology applications. There is little information about their use as probiotic agents, and most reference to probiotic *Brevibacillus* species is as former *Bacillus* species. Their original habitat is not the intestinal tract. A strain of *Brevibacillus brevis* was studied for its use for biocontrol of plant pathogens due to their antimicrobial production (Edwards and Seddon 2001). Another example of the use of *Brevibacillus* is with a strain (HPD31) of the species *choshinensis* used as an efficient producer of recombinant human epidermal growth factor (Miyauchi and others 1999). This strain also serves to convert biologically inactive multimers of epidermal growth factor into biologically active monomers.

Though published literature substantiating health effects of sporeforming "probiotics" is sparse, a variety of products which contain sporeformers are available commercially

Efficacy of *Bacillus*, *Sporolactobacillus*, and *Brevibacillus*

Recently, the term probiotic was defined as "live microorganisms administered in adequate amounts which confer a health effect on the host" (FAO/WHO 2001). Implicit in this definition is that, for the term "probiotic" to be used, a health effect must be demonstrated. Although the published literature substantiating health effects of sporeforming "probiotics" in humans is sparse, a variety of products for human consumption which contain sporeformers are available commercially (Table 1). In some countries, *Bacillus*-based probiotics have been successfully introduced by pharmaceutical companies. For example, Hanmi Pharmaceutical Co., Ltd. in China has a *Bacillus* probiotic marketed as a therapeutic drug with clinical evidence and full regulation by the federal authorities. On the other end of the spectrum are undefined mixtures of "soil organisms" sold as dietary supplements by a variety of companies, which presumably contain sporeformers. The following discussion will highlight some publications documenting physiological effects in humans of sporeforming bacteria. A review summarizes the use of a commercial spore-containing probiotic product, Enterogermina, as an antidiarrheal probiotic (Mazza 1994). Studies aimed at animal feed applications are not the focus for this discussion.

Initial efforts to document a physiological impact of probiotic bacteria often focus on the following three criteria: (1) inherent characteristics of strains that would enable intestinal tract survival, (2) the fate of the fed bacterium, and (3) the impact of consumption of the live bacterium on intestinal flora. (It should be noted, however, that effects beyond an impact on intestinal flora, and at extraintestinal sites, have been documented for many probiotic strains [Reid and others 2003]). A few such studies have been done with sporeformers. Hyronimus and others (2000) evaluated the acid and bile tolerance of vegetative cells of 13 strains of *Sporolactobacillus, Bacillus laevolacticus, Bacillus racemilacticus,* and *Bacillus coagulans* in the vegetative state. They found that only *Bacillus racemilacticus* and *Bacillus coagulans* tolerated oxgall above 0.3%. Strains of *Bacillus laevolacticus* and *Sporolactobacillus* tolerated pH 3 for 3 h, but none of the 13 strains evaluated survived pH 2.0 for 3 hr. Although results suggest that none of the strains evaluated have the necessary ability to survive under conditions present in the human gastrointestinal tract, these studies were conducted on vegetative cells. This study did not test spores which would presumably tolerate such conditions well. However, the 3-h incubation time used in this study is longer than would be likely to be experienced during gastric transit. Incubation times of 30 min to 1 hr are more realistic. Spinosa and others (2000a) studied the fate of *Bacillus subtilis* and *Bacillus clausii* spores after intragastric inoculation into mice. They determined that, although spores survived gastrointestinal transit, their levels declined exponentially to negligible numbers in less than 1 wk and no significant level of vegetative cells could be recovered from the ileum, colon, or feces (vegetative cells were recovered up to 72 h). The authors concluded that the spore form of the *Bacillus* must mediate any probiotic effect. However, Casula and Cutting (2002) tested the ability of *Bacillus subtilis* spores to germinate in the intestinal tract of mice. Using a chimeric gene expressed only in vegetative cells of *Bacillus subtilis* as the basis for their assay, they documented the presence of vegetative cells after feeding of spores. This supports the conclusion that germination of *Bacillus subtilis* in the intestine does occur, and may in fact mediate a probiotic effect. In a different approach, Hoa and others (2001) studied the behavior of *Bacillus subtilis* spores administered by intragastric gavage to mice. They did not differentially count spores and vegetative forms and checked the presence of spores only up to 96 hr after administration. They found that in some experiments the number of spores was larger than expected from the size of the inoculum. Their results suggest that limited germination of spores into vegetative forms could occur. Jadamus and others (2001) demonstrated germination of *Bacillus cereus* var. *toyoi* spores in intestinal samples from both broiler chickens and suckling piglets, and concluded that germination of spores was a necessary prerequisite for its possible probiotic effects. Taken together, these studies suggest that some germination may occur *in vivo*, but still unresolved is the role of these different cell states in mediating physiological interactions *in vivo*.

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Impact on fecal microflora. Regarding the impact of sporeformers on fecal microflora, Adami and Cavazzoni (1999) studied the effect of *Bacillus coagulans* CNCM I-1061 on the profile of bacteria in feces in a piglet model. They found that *Bacillus coagulans* consumption (~10^{11} cfu/kg feed, 50% spores) increased aerobic and anaerobic sporeformers, decreased lactococci, enterococci, anaerobic cocci, and fecal coliforms over time. Hosoi and others (1999) tested the impact of *Bacillus subtilis* (natto) on the fecal flora of mice. Spores did not affect fecal *Enterobacteri-*

aceae nor *Enterococcus* spp., regardless of diet groups, but differences in *Bacteroidaceae* and *Lactobacillus* were observed. No changes were observed when autoclaved spores were administered. Studies on the impact of spores on the fecal flora of humans have not been reported.

Production of antimicrobials. The production of antimicrobials is considered to be a pathogen-inhibiting mechanism exhibited by probiotic bacteria, and such compounds have been shown to be produced by some sporeformers. Dozens of different peptide antibiotics exhibiting antagonism against a broad spectrum of microbes have been identified from the *Bacillus* genus. Most are not useful clinically, however, although polymyxin produced by *Bacillus polymxya* is a notable exception to this trend. Lee and others (2001) documented that *Bacillus polyfermenticus* produced a bacteriocin, polyfermenticin SCD. This heat labile, proteinase K-sensitive compound primarily inhibited other *Bacillus* species. Pinchuk and others (2001) identified two distinct heat-stable, protease-resistant anti-*Helicobacter pylori* activities produced by *Bacillus subtilis* 3 *in vitro*. One activity was identified as amicoumacin A. However, it is not clear how these antimicrobials could be produced in the stomach where *Helicobacter* is found and where evidence suggests that the vegetative cell would be destroyed. Therefore, their *in vivo* relevance remains to be demonstrated. Hyronimus and others (1998) identified an inhibitory substance, coagulin, produced by *Bacillus coagulans* I_4. This bacteriocin-like compound was plasmid-linked, heat-stable, protease-sensitive, and exhibited both bacteriocidal and bacteriolytic action against related and unrelated bacteria (*Leuconostoc, Oenococcus, Listeria, Pediococcus,* and *Enterococcus*). This discovery was followed up by Le Marrec and others (2000), who characterized coagulin by N-terminal sequencing of the purified 44-amino acid peptide and found it quite similar to pediocins produced by *Pediococcus acidilactici*, differing by only a single C-terminus amino acid. Furthermore, high homology was identified between the plasmid-encoded coagulin gene in *Bacillus coagulans* and the genes encoding the pediocin peptide from *Pediococcus*. Some good basic experimentation has been conducted on *Bacillus* bacteriocins, but no studies documenting the effectiveness of these bacteriocins produced *in vivo* on pathogen inhibition have been conducted.

Studies on the impact of sporeformers on promoting human health are intriguing but scanty

Animal models. Demonstrating a physiological benefit in animal models is another important step in establishing probiotic efficacy. Sorokulova and others (1997) tested several commercial products (Biosporin from Ukraine, Subalin from Ukraine, Bactisubtil from Yugoslavia, and Cereobiogen from China) containing *Bacillus* species for *Campylobacter* inhibition. *In vitro* inhibition of several *C. jejuni* and *C. coli* strains was observed by Biosporin products containing *Bacillus subtilis* or *Bacillus licheniformis* and by the *Bacillus subtilis*-containing Subalin product. Neither the Bactisubtil nor the Cereobiogen products demonstrated *in vitro* inhibition of the *Camplylobacter* strains. Only Biosporin and Subalin were tested in the mouse model of *Camplylobacter* infection. The authors concluded that a single preventative oral dose of either of these products caused a protective effect; however, the endpoints for protective effect and the statistical significance of their results were not clear in the paper. In a model of *Vibrio* infection in rabbits, Hattori and others (1965) demonstrated that *Bacillus coagulans* P-22 inhibited *Vibrio*-induced changes (histological appearance and exudates accumulation) in intestinal tissue.

Animal feed supplements. Most other papers on the role of sporeformers in animals are focused on their use as animal feed supplements. Although this is not the focus of this paper, their effectiveness as such is noteworthy. For example, Cavazzoni and others (1998) tested the effect of *Bacillus coagulans* as facilitators of growth and efficient food conversion in chickens in 2 independent studies. The studies compared *Bacillus coagulans* or virginiamycin supplements to the diet to a control group with no supplements. In the first study, the *Bacillus coagulans*-treated group outperformed the control group and was equivalent to or better than the antibiotic-fed group with mean body weight and daily weight gain from day 7 through the end of the study at day 49. Feed intake was unchanged. In the second study, the same results were observed, except statistically significant changes did not occur until day 33 of the study. Similar results are the basis for several commercial animal supplement products. Their effectiveness resulting in cessation or reduction of use of antibiotics in animal feeds would be important.

Physiological differences between spores and vegetative cells suggest that probiotic effects differ based on physiological state

Impact on immune function. Modulation of immune function is a postulated mechanism of action of probiotic bacteria, including sporeformers. Heat- or formaldehyde-inactivated *Bacillus* species exhibited immunopotentiating activity in mononuclear leukocytes isolated from humans (Prokešová and others 1994). But a few studies have been published on immune enhancement by orally consumed sporeforming bacteria. Muscettola and others (1992) demonstrated that the Enterogermina product containing different strains of *Bacillus clausii* spores administered to ice increased interferon production *ex vivo* by stimulated peritoneal and spleen cells. Ciprandi and others (1986) evaluated the effects of *Bacillus clausii* (formerly designated *Bacillus subtilis*) spores and vegetative cells on mitogenic T-cell proliferation and mitogen-induced lymphokine production by mononuclear cells isolated from healthy volunteers. Only vegetative cells stimulated mitogen-induced mononuclear cell proliferation. Neither spores nor vegetative cells modified interleukin-2 or γ-interferon production. Vacca and others (1983) tested immunomodulating effects of *Bacillus clausii* spores (Enterogermina) in an open label study of 11 multiple myeloma patients serving as their own controls. Spores were administered 3 ×/d (6×10^9 spores/d) for 21-d periods, alternated with a 21-d rest period following 7-d cytostatic therapy. Improved cell-mediated immunity parameters, including response of E-rosettes and monocyte chemotaxis, was observed after spore treatment. Furthermore, 4 patients experienced a decrease of recurrent respiratory infections occurring before immunomodulating treatment. No side effects were reported to the spore treatment. Kozuka and others (2000) and Goto and others (2000) have used engineered strains of *Brevibacillus* as delivery agents for mucosal adjuvants.

Human studies. Published human clinical trials with oral consumption of sporeformers are few. One study on the impact of sporeformers on reduction of blood lipids was reported. This study was conduct-

ed with *Bacillus coagulans* (marketed as "Lactobacillus sporogenes"). Although there are 2 retrievable references, these papers report the same data (Mohan and others 1990a, b). The data were derived from an open-label, nonrandomized study of 17 hyperlipidemic patients. Patients received 2 tablets 3 x/d for a total daily administration of 3.6×10^8 spores for 12 wk. Reductions in total cholesterol (330 to 226 mg %) and LDL (267 to 173 mg %) were recorded, but no dietary control was conducted. No adverse incidents were reported. These results suggest interesting pilot study results, but cannot be considered conclusive for a role of *Bacillus coagulans* in reducing serum lipids in hyperlipidemic patients. This is the only retrievable study on "Lactobacillus sporogenes" in humans.

To date the FDA has not granted GRAS status for any sporeformer probiotic application

The effect of *Bacillus subtilis* on patients with slow or static urinary flow (as a biomarker of urinary tract infection risk) was studied (Meroni and others 1983). The study was a randomized, placebo-controlled trial of 80 elderly adults (mean age 75.5 y) treated daily for 6 mo with 2 vials (number of spores per vial was not indicated) of *Bacillus subtilis* ATCC 9799 spores given orally. During the 5th and 6th months, a statistically significant reduction in the number of patients with at least 1 positive urine culture during each month and consecutive altered sediment leukocyte counts (leukocytes >106/24 h) was observed.

The studies published on the impact of sporeformers on promoting human health are intriguing but scanty. It can be concluded that some strains of sporeformers (1) produce antipathogenic compounds, (2) production of these antipathogenic compounds may result in an antipathogenic response in some animal models, (3) the resistance of spores to environmental stress ensures that these bacteria can pass through the gastric barrier alive and can remain viable for long periods in commercial preparations without refrigeration, (4) vegetative cell or cell components react to immune cells enhancing immune response, but the extent of this response in humans has not been adequately substantiated, (5) no adverse incidents have been reported in published human trials, and (6) retrievable references on controlled human trials reporting the role of sporeformers in human health are few and are limited to *Bacillus* species.

Mechanisms of action. The fundamental physiological differences between spores and vegetative cells suggest that probiotic effects may be mediated by mechanisms which differ based on physiological state. Most research on mechanisms of efficacy of probiotics in general has been conducted on nonsporeformers. Preliminary evidence indicates that spores of *Bacillus subtilis* do not associate closely with intestinal cells, nor do they elicit immune response (Tompkins, unpublished data, 2002). Hosoi and others (1999) have shown that autoclaved spores do not influence the intestinal microflora in mice. Such studies have led to the hypothesis that probiotic efficacy of spores may be mediated through metabolites or enzymatic activities. The *Bacillus* species produce proteases (for example, subtilisin), which aid digestion and reduce allergenicity. They are also said to produce vitamin K2 (Hosoi and Kiuchi 2003). The catalase and subtilisin produced by *Bacillus* species have been shown *in vitro* to promote *Lactobacillus* growth (Hosoi and others 2000). However, some evidence of spore germination *in vivo* has been published in animal models (Casula and Cutting 2002; Hoa and others 2001; Jadamus and others 2001). It is not possible at this time to conclude about the role of the spore or vegetative states in mediating probiotic function. In studies conducted in chickens fed dietary *B. subtilis* natto for 28 d (Samanya and Yamauchi 2002), intestinal histologies (such as villus height, cell area, and cell mitosis) were altered relative to the controls. Additionally, it was observed that blood ammonia was depressed. It was hypothesized that that intestinal function was activated by the depressed blood ammonia concentration. Other evidence for physiological activity came from a study focused on antigenotoxicity activity of 16 *Bacillus* strains (Caldini and others 2002). Deactivation of 4-nitroquinoline-1-oxide in a short-term bacterial assay was demonstrated. Mechanisms of action of sporeformers as probiotics remain to be defined, but may include metabolic activities of the microbe, secretion of antimicrobials, and immunomodulation. Certainly more research is needed on the mechanisms of probiotic action of sporeformers.

There have been no toxicity data published on the various *Bacillus* species with respect to their use as human probiotics

Safety of sporeforming probiotics

The commercial uses of *Bacillus* species are numerous and include the production of antibiotics, amino acids, enzymes, and fermented beans. This fact helps establish a cornerstone for safety evaluation of these bacteria. However, the use of *Bacillus* as a probiotic involves direct consumption of high concentrations of viable microbes. It is rare for any substance to be considered safe for any use. The differences between consumption of certain *Bacillus* species as part of a fermented food and consumption of other species of *Bacillus* at concentrated doses as a biotherapeutic must be considered fundamentally different uses with safety evaluation ramifications. With the exception of *Bacillus anthracis* and *Bacillus cereus*, *Bacillus* species have not generally been considered pathogenic. Indeed, spores of *Bacillus* are regularly consumed by animals and man inadvertently through the food and feed supply, and in some fermented foods. The occurrence of *Bacillus* species (*subtilis, licheniformis, cereus, circulans, thuringiensis, sphaericus, badius, firmus, megaterium, mycoides,* and *sphaericus*) and *Brevibacillus laterosporus* as components of fermented soy or locust beans was demonstrated with random amplified polymorphic DNA analysis (Sarkar and others 2002), documenting the association of these sporeformers with fermented foods. However, there have been many recent reports of infections associated with *Bacillus* species, and these cases are noteworthy when considering the safety of *Bacillus* as a probiotic. While food poisoning is perhaps the greatest risk of *Bacillus* species, there are reports of serious local and opportunistic systemic infections and abortions by these microorganisms (SCAN 2000a). There is sufficient evidence to suggest that probiotic products based on sporeforming bacteria undergo more rigorous evaluation before marketing. Many of these products may prove not to be a threat to the health and well-being of the consumer, but insufficient characterization makes

this determination difficult. To date, the only commercially available *Bacillus* that has been investigated in safety and toxicity studies and shown to be safe is *Bacillus subtilis* and even in this case the data have not been widely published.

A misconception perpetuated by some manufacturers and retailers is that *Bacillus* species are Generally Recognized as Safe (GRAS) by the Federal Drug Administration (FDA) in the United States of America. While it is true that some *Bacillus* species are recognized as GRAS for specific applications such as enzyme production, to date the FDA has not granted GRAS status for any sporeformer probiotic application. While probiotic bacteria of the genera *Lactobacillus* and *Bifidobacterium* have been subjected to rigorous testing for acute and chronic toxicity (Donohue and others 1993) and panels of experts have reveiwed relevant data and concluded their safety for use as probiotics (Adams and Marteau 1995), there have been no toxicity data published on the various *Bacillus* species with respect to their use as human probiotics. The production of emetic and enterotoxins in clinically relevant strains of *Bacillus* has been more extensively studied. Significant numbers of toxigenic strains of *Bacillus subtilis, Brevibacillus laterosporou*s, and others have been reported. Effective toxin detection assays, which should be applied to sporeformers used in probiotic products (Beattie and Williams 1999), have been developed. Although adherence to intestinal epithelial cells is frequently touted as a valuable characteristic of probiotic strains, including sporeformers, studies have suggested that *Bacillus* adhesion is a virulence mechanism (Andersson and others 1998; Rowan and others 2001).

The safety of *Bacillus subtilis* and *Bacillus amyloliquefaciens* has been reviewed (de Boer and Diderichsen 1991). This review specifically examined published incidences of *Bacillus* infections. These infections were not due to direct ingestion of *Bacillus*, but from other sources. The findings showed that infections most frequently appeared in people with a history of endocarditis, who were immunosuppressed or had recently undergone surgery. While the paper also admits that reported cases of food poisoning by *Bacillus subtilis* are very low, it points out that exact and reliable figures are hard to obtain. This is because hospitals do not necessarily differentiate between *Bacillus cereus* and other species of *Bacillus* as agents of food poisoning. *Bacillus* species have been associated with nosocomial bacteremia (Richard and others 1988).

Cases of infection resulting from *Bacillus* probiotic consumption have been reported. Oggioni and others (1998) reported septicaemia caused by *Bacillus subtilis* strains from a probiotic preparation used by an immunocompromised patient. Spinosa and others (2000b) examined 2 published examples of *Bacillus* infections which may have been linked to a commercial probiotic preparation. The infectious agent found in one case of cholangitis in France in 1996 and one case of recurrent septicemia in Italy in 1998, were indistinguishable from a *Bacillus* strain found in an Italian probiotic product *(Bacillus clausii*). However, the authors could not confirm a causal role of the Italian probiotic in the infections. Both infections occurred in immunosuppressed patients. The French patient had undergone a kidney transplant and the Italian patient was undergoing chemotherapy. The authors recommended the development of a public database for all alimentary and probiotic strains where characteristics relevant for identification and typing could be deposited.

Some efforts to characterize the antibiotic resistance of *Bacillus* strains used as probiotic products have been made. Both Ciffo (1984) and Mazza and others (1992) evaluated 4 strains of *Bacillus clausii* which comprise the commercial product Enterogermina for their resistance to therapeutic antibiotics. Mazza and others (1992) went on to test the stability and transferability of these resistance traits. Stable resistance to cephalosporins, macrolides, and quinolones was observed, but transfer of these resistance phenotypes to other bacteria *in vitro* or *in vivo* was not detected.

An important aspect of establishing safety of a probiotic is proper taxonomic characterization of the bacteria in the product. Green and others (1999) tested 2 commercial products (Enterogermina and Biosubtyl) and showed that neither was composed of *Bacillus subtilis* as claimed by the manufacturers. This was based on several key phenotypic characteristics (amylase activity and alkaline growth) and total 16S rDNA sequences. The Enterogermina strain most closely aligned with the *Sporolactobacillus* group (*Bacillus alcalophilus* subgroup) and the Biosubtyl strain was most closely related to *Bacillus pumilus*.

It is imperative that sporeforming probiotic product be accurately represented to the consumers. One approach is for industry to develop and use objective and science-based guidelines for commercial

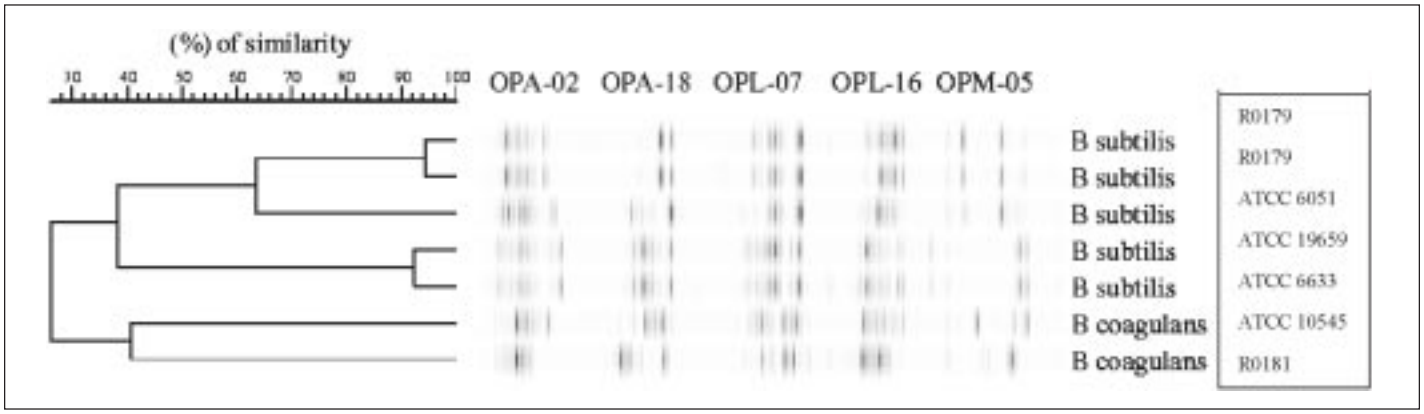

Figure 2—Randomly amplified polymorphic DNA-polymerase chain reaction profiling [with dendrogram] is an example of one technique that can be used to establish strain identity, which is a crucial early step in the manufacturing of a well-characterized probiotic. It is also an essential tool to ensure that the microbe has not changed during storage or production. Note that there are 2 profiles shown for R0179: the first lane represents the strain before production (mother culture) and the second lane represents the strain after industrial production (commercial product). Image courtesy of Dr. Denis Roy, Agriculture & Agri-Food Canada, St. Hyacinthe, QC, Canada.

products. Alternatively, it is likely that governments will recognize the need to impose more stringent regulations regarding these particular microorganisms. The following list of recommendations is based partially on the current guidelines proposed researchers (Donohue and Salminen 1996; Przyrembel 2001) and world organizations (SCAN 2000a, b; FAO/WHO 2001). It is suggested that they be considered the minimal safety information for companies to bring sporeforming bacteria to market as probiotics:

1. Each strain of bacterium in the product must be isolated, named, and taxonomically identified. Speciation must be unequivocally established using the most current, valid methodology. Generally, a combination of gene-based and phenotypic techniques are necessary. 16s rRNA gene sequencing with comparison to type strains from a recognized culture collection such as ATCC, the European culture collections (DSMZ, LMG, CIP, NCIMB) or Japanese collection (IAM) is the best available approach for most microbes. A strain-specific pattern derived from total chromosomal (for example, pulsed field gel electrophoresis, randomly amplified polymorphic DNA-polymerase chain reaction), or rDNA must be established (Figure 2). If the identity of the strain or strains of bacteria in a product is in question, no conclusions can be made on its safety.

2. Nomenclature of the bacteria must adhere to the current, scientifically recognized names. Protracted use of older or misleading nomenclature is not acceptable on product labels. Current nomenclature can be ascertained as indicated in the earlier section of this paper on nomenclature.

Announcement on a Validation List, which validates bacterial names published elsewhere. The Validation List is published in the Intl J of Syst and Evolutionary Microbiol.

3. Sufficient *in vitro* characterization of each strain of bacteria should be conducted, including: antibiotic resistance profile, production of emetic or enterotoxins, gastric acid resistance, and bile resistance. Bacterial strains that demonstrate transferable antibiotic resistance should not be employed. A detailed scheme for testing toxin production has been already recommended by the Scientific Committee on Animal Nutrition (SCAN 2000b). Any strains capable of producing toxins must not be used as a probiotic.

Bacteria should be characterized for the presence of plasmids or transferable DNA vectors. *Bacillus* plasmids have been shown to mediate interspecies transfer of antibiotic-resistance (Koehler and Thorne 1987), and this risk must be avoided.

In many cases, use of probiotics does not require premarket approval, but this does not constitute permission to market uncharacterized products with unsubstantiated safety and efficacy

4. A review of safety should be conducted by an independent 3rd party panel of experts qualified in the field. Depending on the genus and species used, the intended dose, and the target consumers, safety characterization may be conducted as follows. The ability to adhere to, invade, and modulate the immune system of appropriate human cell lines should be evaluated. Strongly adherent or invasive strains of bacteria in vegetative or spore form should not be used as probiotics. Acute toxicity and embryo toxicity studies would contribute to understanding of safety. Testing of each strain in concentrated form (in both vegetative and spore state), and the final product, on at least one mammalian species may be needed. Repeated dosage chronic toxicity studies, for a minimum of 9 mo, should be performed on each strain in concentrated form (in both vegetative and spore state) and the final product on at least 2 mammals, preferably a rodent and one larger species (for example, pig, rabbit, or cat).

5. Label and marketing literature must list contraindications for *Bacillus* species or sporeforming bacteria, including specific references to patients or consumers who are immunosuppressed as a result of HIV infection, chemotherapy, or allograft therapy.

6. Marketing literature or product label should provide:

(a) Indications for use supported by clinical evidence.

(b) Clear definition of the genus, species, strain, and concentration of each bacterial component of the product.

(c) An adverse reaction reporting telephone number must be clearly indicated on the product label.

Conclusions

The probiotic industry is in a growth phase, with enhanced activity in traditional functional food use and also expanded interest in use for specific therapeutic indications. In general, current government legislation does not provide definitions regarding the suitability of various bacteria for food and supplement use. In most countries, products marketed as pharmaceuticals must meet premarket criteria for efficacy and safety. This approach provides consumers with assurances on product quality. In many cases, use of probiotics in dietary supplements (in the U.S.) or foods does not require premarket approval, but this does not constitute permission to market uncharacterized products with unsubstantiated safety and efficacy. The responsible manufacturer must consider the standard for safety as essentially the same, regardless of the marketing niche for the product. In fact, safety for a food or supplement could be considered more stringent, in that risk/benefit assessment is acceptable for pharmaceutical products but not for foods or supplements. Many companies are promoting the therapeutic use of *Bacillus* and other sporeforming bacteria in concentrated dosages, but these companies should not compromise on safety assessments for their products. Companies are leveraging the temperature resistance of the spores in order to make "shelf-stable" label and marketing claims. Some of these companies are deliberately misusing nomenclature, such as referring to their *Bacillus coagulans* strains as "*Lactobacillus sporogenes*," to take advantage of the more predominant scientific literature that exists for the lactobacilli. In the end, the consumer's concern is that safe, efficacious products are available to them. In the case of many sporeforming bacteria sold as probiotics, their concerns have not been adequately addressed.

In contrast, probiotic research with the lactobacilli and bifidobacteria has progressed quite steadily. Numerous articles reviewing the safety (Borriello and others 2003) and efficacy (Guarner and Malagelada 2003) of this type of probiotic have been published in recent years. A Medline search of articles using the keyword "probiotic" and limited to clinical studies over the past 10 years resulted in 123 references. Not one included the terms "*Bacillus*, *Sporolactobacillus*, or *Brevibacillus*." Much remains to be done to substantiate sporeformers as probiotics.

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