Importance of the gastrointestinal life cycle of *Bacillus* for probiotic functionality

M. Bernardeau^{1,2} · M. J. Lehtinen³ · S. D. Forssten³ · P. Nurminen³

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Abstract *Bacillus* spp. are widely used in animal production for their probiotic properties. In many animal species, feed supplementation with specific *Bacillus* strains can provide numerous benefits including improvement in digestibility, the gut microbiota and immune modulation, and growth performance. Bacilli are fed to animals as spores that can sustain the harsh feed processing and long storage. However, the spores are metabolically quiescent and it is widely accepted that probiotics should be in a metabolically active state to perform certain probiotic functions like secretion of antimicrobial compounds and enzymes, synthesis of short chain fatty acids, and competition for essential nutrients. These functions should become active in the host gastrointestinal tract (GIT) soon after digestion of spores in order to contribute to microbiota and host metabolism. Considering that bacterial spores are metabolically dormant and many health benefits are provided by vegetative cells, it is of particular interest to discuss the life cycle of *Bacillus* in animal GIT. This review aims to capture the main characteristics of spores and vegetative cells and to discuss the latest knowledge in the life cycle of beneficial *Bacillus* in various intestinal environments. Furthermore, we review how the life cycle may influence probiotic functions of *Bacillus* and their benefits for human and animal health.

Keywords *Bacillus* · Spore · Vegetative cells · Germination · Probiotic · Gastrointestinal tract

Introduction

Recently, the International Scientific Association for Probiotics and Prebiotics (ISAPP) emphasised the need for the clarification of the definition of the term probiotic. In a consensus opinion published in 2014, the expert panel reinforced the importance of viability of probiotic microorganisms by defining them as: “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al. 2014). In their opinion, viability is being implicitly linked to some mechanisms of probiotic action suggesting that the probiotic should be metabolically active at the site of action i.e. in the gut. Traditionally a large variety of microbial species used as probiotics in food and feed are already metabolically active even before entering the host, including yeast (*Saccharomyces*) and non sporulating bacteria (*Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, and *Streptococcus*). For decades, scientists have investigated solutions and technologies to improve the survival rate and the delivery of probiotics to the gastrointestinal tract (GIT) given the harsh environmental conditions in the feed process and the upper GIT. Strategies that have been used include sub-lethal stresses (Nguyen et al. 2016), use of protectants (Riaz and Masud 2013), drying technology (Broeckx et al. 2016), encapsulating materials (Opara 2017; Huq et al. 2013) and immobilisation techniques (Mitropoulou et al. 2013) that improve stability, survival, long-term preservation and biotherapy of probiotics. However, by nature bacterial spores don’t require these technologies to resist the food and feed processing effects.

✉ M. Bernardeau
marion.bernardeau@dupont.com

✉ P. Nurminen
paivi.nurminen@dupont.com

¹ DuPont-Danisco, Industrial Biosciences, Animal Nutrition, Marlborough, UK

² Normandy University, ABTE, 14032 Caen, France

³ DuPont Nutrition and Health, Kantvik, Finland

The sporulating probiotic bacteria (*Bacillus*, *Clostridium*) are highly resilient and retain their viability during distribution and storage (Cutting 2011), but they need to regain their metabolic activity after entering the host to provide benefits. Thus, it is of interest to understand the dynamics of *Bacillus* spp. germination in the gut including the fate of ingested *Bacillus* spores and the ratio of vegetative cells and spores in the different gut sections across different hosts, the length of time that the *Bacillus* persist in the gut after their withdrawal from the diet, and the influence of GIT physiology and *Bacillus* strain.

While the life cycle of pathogenic *Bacillus* in human GIT has been extensively researched (Hutchison et al. 2014; Narula et al. 2016), little has been published on the life cycle of beneficial spore formers during the last decade. The aim of this review is to capture the main characteristics of spores and vegetative cells and to discuss the latest knowledge in the life cycle of beneficial *Bacillus* in various intestinal environments. Furthermore, we review how the life cycle may influence probiotic functions of *Bacillus* and their benefits for human and animal health.

Characteristics of spores and vegetative cells

Bacillus cells have two distinct morphologies, endospore and vegetative cell, depending on the environmental stimuli (Piggot and Hilbert 2004). Endospores are the most durable type of cells found in nature, and represent a survival strategy of the bacteria. *Bacillus* endospores, or more traditionally referred to as *Bacillus* spores, have been isolated from amber that is more than 25 million years old, demonstrating the metabolic dormancy and protective capability of spores (Cano and Borucki 2012). The spores protect the bacterium by attaining a highly defensive state that is resistant to environmental

conditions such as extreme temperature, radiation, pH, pressure and toxic chemical agents that would harm the vegetative form (Pedraza-Reyes et al. 2012). Some parts of the GIT represent a toxic environment for *Bacillus* with anoxia, low pH, and bile salts, along with an extremely high concentration of commensal bacteria (reaching 10^{12} g⁻¹ of faecal content in the colon) that compete for nutrients and ecological space. These conditions challenge the growth of the vegetative bacterium, yet the spore would be inert to these conditions and transit unimpeded through the GIT.

The outermost structure of endospores, the exosporium, consists of proteins, lipids and carbohydrates that provide hydrophobic characteristics to the spore and make it insoluble. The extremely low permeability of the inner membrane prevents both hydrophobic and hydrophilic molecules from entering the core. Bacterial spores contain a high concentration of dipicolinic acid (DPA), the exact content of which varies between different species yet ranges from 5 to 15% in dry weight. The unique and intrinsic composition of *Bacillus* spores provides high tolerance to the acidic conditions in the stomach upon ingestion as described in Table 1.

The spore will germinate in the presence of germinants, nutrients or non-nutrients (Swick et al. 2016) and other favorable environmental conditions. Nutrients activate the germinant receptors causing the release of ions including Ca²⁺ and DPA from the spore core. In addition to nutrients, spores are germinated by other agents, including lysozyme, salts, high pressures and cationic surfactants such as dodecylamine (Setlow 2003). Binding of germinants to the receptors present on the inner membrane triggers the release of the DPA, allowing water to enter the spore core. This re-hydration activates spore enzymes that hydrolyse the spore cortex, allowing the spores to start germination. Cortex hydrolysis and subsequent cortex expansion allows

Table 1 Differences between the resistance profiles of *Bacillus* spores and vegetative cells against adverse environmental and gastrointestinal tract conditions

Condition	Vegetative cells	Spores	References
Extreme pH	Inactivated with variable kinetics depending on the specific nutrients composition and strains used	Not inactivated between 1.0 and 5.0 in gastric medium	Clavel et al. (2004)
Stomach acid	Highly sensitive	Trigger germination	Ciffo et al. (1987)
		No effect on spore	Duc Le et al. (2003a)
Bile salts	Highly sensitive <1 mmol ⁻¹ Bacteriocidal on VC	No effect on spores	Hyronimus et al. (2000)
		Modest inhibition of germination	Duc Le et al. (2003a)
Intestinal conditions	Suitable growth environment in the absence of intestinal microbiota exerting competitive pressure	Suitable growth environment in the absence of intestinal microbiota exerting competitive pressure	Ceuppens et al. (2012d)

full hydration of the spore core. DPA is rapidly released during germination, but its function remains uncertain. Studies have shown that spores lacking DPA have significantly increased water, and decreased heat and H₂O₂ resistance (Balassa et al. 1979). In addition, it is possible that Ca²⁺-DPA released from one spore may stimulate the germination of other neighboring spores (Setlow 2003). This enables the restart of metabolism and macromolecular synthesis in a process termed outgrowth, eventually leading to the resumption of vegetative growth (Setlow 2003). However, when the dormant spore breaks hibernation and begins to be metabolically active, it also loses resistance to environmental stress. Thus, the structure of the spores and vegetative cells have adapted to specific environments at different stages of the life cycle that are controlled by sophisticated molecular mechanisms. The sporulation capability of probiotic *Bacillus* would be a perfect advantage but only if germination occurs fast enough to generate enough live cells that could reach the appropriate location within the gut and display beneficial effects.

Life cycle of *Bacillus* in various GITs

Globally, available data indicates that *Bacillus* spores may have similar life cycles in mammals (mice, pigs) and avian species (chicken). They can germinate in variable proportions and at different locations depending on the physiological characteristics encountered in the gut as described in further detail below for each model.

Animal models

Laboratory animal models, mainly mice (Table 2), have been used to study the *Bacillus* life cycle in more depth. It has been shown that the spores administered by the intra-gastric route to mice could not germinate and on the other hand, ingested spores that did not germinate prior to reaching the large intestine remained metabolically dormant during the rest of the transit (Table 2; Spinosa et al. 2000). These results suggest that the stressful conditions encountered during the upper digestive tract may be the key for triggering the germination process and that the time window for germination is limited. One of the key triggering factors for sporulation is the low pH in the stomach (Ciffo et al. 1987) that, conveniently, activates the spores before they reach the small intestine which is rich in nutrients. Further evidence on the importance of the small intestine as the main sporulation site was shown in a mice study that used recombinant *Bacillus subtilis*. The authors showed that the spores, upon exiting the stomach, can germinate, proliferate, and then resporulate in the small intestine of mice (Tam et al. 2006).

Despite the important use of probiotic *Bacillus* in animal nutrition, only a few studies have been published on the germination potential of *Bacillus* spores in farm animals (Table 2). In chickens, it was observed that 10–90% of spores germinated in the small intestine (Latorre et al. 2014) (Table 3). In vitro, crop and intestinal models have shown that up to 90% of the spores germinate within 60 min (Latorre et al. 2014). In addition to highlighting that *Bacillus* have adapted to different hosts, Hong et al. (2009) reports that there is a mixture of spores and vegetative cells in the small intestine and that upon exiting the stomach or crop *Bacillus* are mostly still in their spore form. However, it seems that the germination is not systematic and that the host, the intestinal location of germination as well as the bacterial species may be crucial contributing factors for the survival and metabolic activity of vegetative cells along the GIT.

There are species and strain specific differences in the persistence of *Bacillus* strains in the GIT. In mice, persistence of different species ranges from 7 to 27 days indicating differences in the life cycle of the *Bacillus* strains (Tam et al. 2006). In another study using mice, all *Bacillus cereus* tested strains appeared to be more persistent than *B. subtilis*, *B. pumilus* and *B. clausii* (Duc Le et al. 2004b; Table 2). The rationale for these findings may be found in metabolic differences between species. Some *Bacillus* species are facultative anaerobes with anaerobic respiration and fermentative growth capabilities (Clements et al. 2001) and could survive in various GIT locations independent of the O₂ conditions. On top of nutritional and aerobic specificities occurring across species, differences in germination are the result of the genetic makeup, resulting in significant differences between various strains belonging to the same species and the heterogeneous behaviour of individual cells within an isogenic population (Wells-Bennik et al. 2016).

In addition to strains, the host appears to impact the *Bacillus* life cycle. When comparing work done in mammals like mice (Table 2) and pigs (Table 3) and in chickens (Table 3), it seems that in avian species, the vegetative cells are found in every part of the GIT whereas in mammals, they are mainly recovered in the distal GIT as for mice (Cartman et al. 2008) and for pigs (Leser et al. 2008). Interestingly, the work done by Cartman et al. (2008) in mice suggests that the maturity of the GIT may be important in the germination process of spores. In addition, it seems that the resident intestinal bacteria (mainly from the large intestine) may limit or suppress the outgrowth of the vegetative cells.

Studies also highlight that survival and persistence of *Bacillus* in the GIT seems to be related to the inoculated form and dose in a species-specific manner. Comparative analysis of survival rates of ingested spores versus

Table 2 Overview of studies of germination and overgrowth of sporulated *Bacillus* added to mice diet or administered intragastrically)

Animal Target	Strain	Dose, Physiological status	Germination (G) or not	Results	References
Mice	<i>B. subtilis</i> (natto)		G	Enhancement of the growth of <i>Lactobacillus murinus</i> seemed to be linked to metabolites produced during germination and (or) outgrowth of spores of the strain Natto	Hosoi et al. (1999)
Mice	<i>B. subtilis</i> MO 1099 <i>B. clausii</i> (Enterogermina®-Sanofi)	1×10^9 spores/animal intragastric	No G	Elimination of SP from the intestine reflecting solely its intake from food (logarithmic phenomenon) No VC: explained by the sensitivity to taurodeoxycholic acid Probiotic effect due to SP only or extra-intestinal life cycle (VC found in mesenteric lymph noodles)	Spinosa et al. (2000)
Mice	<i>B. subtilis</i>	5.87×10^8 SP/mice 2×10^9 SP/mice	Only evidence of G	Only evidence that SP may germinate. SP are not disseminated in significant numbers beyond GIT High persistence rate in the gut that could be ascribed to a small proportion of the SP germination in upper tract of intestinal tract, multiplying once or twice and then resporulating in the distal part of the gut	Hoa et al. (2001)
Mice BALB/c	<i>B. subtilis</i> SC2288 resistant to chloramphenicol	6×10^8 SP/mice	1–12% G	G found in jejunum and ileum using a competitive RT-PCR targeting a genetically engineered chimeric gene <i>ftsH-lacZ</i> only expressed in VC. No VC was found in duodenum	Casula and Cutting (2002)
Mice	<i>B. subtilis</i> SC2362 carries <i>rrnO-lacZ</i> gene as well as <i>cat</i> gene	2.4×10^{10} VC/mice 2.1×10^8 SP/mice	G	% of recovery of VC in feces: 0.00016% of inoculating dose % of recovery of SP in feces: 12% of inoculating dose Germination was found associated to as systemic anti-beta galactosidase IgG response in small intestine	Duc Le et al. (2003a)
Mice	<i>B. subtilis</i> PY79 <i>B. pumilus</i> Biosubtyl ^{NT} <i>B. clausii</i> Enterogermina® <i>B. cereus</i> var. vietnami Subtyl <i>B. cereus</i> Biosubtyl ^{DL} <i>B. cereus</i> Bactilsubtil	10^9 SP/mice per gavage	P	Except the wild-type strain PY79 which rapidly cleared from mouse gut, all other probiotic strains persist until 18 days after inoculation. All <i>B. cereus</i> strains persist longer in the GIT of mice: after 15 days significant numbers are still present	Duc Le et al. (2004b)
Mice C57BL/6	3 strains of <i>B. subtilis</i> <i>B. subtilis</i> PY79; RH103 carries a chimeric gene tetanus toxin fragment C); UL12 with Cm ^r All strains are GMO	2×10^{10} SP/dose on days 1, 2, 3, 24, 25, 26, 49, 50 and 51	G	Declining fecal shed. Strains do not permanently colonize the GIT Based on gene expression in the GIT of mouse Persistence from 15 to 27 days	Tam et al. (2006)
Gnotobiotic rats	<i>B. thuringiensis</i> subsp. <i>israelensis</i>		G		Wilcks et al. 2008

Table 2 continued

Animal Target	Strain	Dose, Physiological status	Germination (G) or not	Results	References
Mice Balb/c AnNHsd	<i>B. polyfermenticus</i> CJ6	10 ⁹ SP/mouse	G P >11 days	VC and SP resist to in vitro simulated gastric juice, bile salt and acid SP germinate and persist in GIT of mice as fecal excretion was 4-fold higher compared to inocula	Jung et al. (2012)
Balb/c inbred mice	<i>B. subtilis</i> HU58 human origin <i>B. subtilis</i> PXN21 (Protexin Ltd) <i>B. subtilis</i> PY79 laboratory strain	1 × 10 ⁹ SP/mice	P of PY79; PXN21 and HU58 is 7, 18 and 21 days respectively	Transit period for <i>B. subtilis</i> varies considerably between different strains Persistence of HU58 and PXN21 is correlated with their abilities to form biofilm	Permpoonpattana et al. (2012)

VC vegetative cell, G germination, SP spore, GIT gastrointestinal tract, P persistence, RT-PCR reverse transcription polymerase chain reaction

vegetative cells of *B. subtilis* in mice revealed that ingestion of only the sporulated form enables sufficient faecal recovery (Duc Le et al. 2004b). The recovery rate of the spores was 75,000-fold higher than the recovery rate of the vegetative cells (Duc Le et al. 2004b) suggesting both germination and proliferation occurred since it is thought that cells must proliferate, at least one cell division before sporulation can commence.

Essential for the life cycle of *Bacillus* is also timely sporulation before exiting the GIT. In the 1970s, conditions encountered in the large intestine were shown to re-activate the sporulation process, requiring both time and energy (Piggot and Coote 1976). This finding is supported by the long persistence time (27 days) of *Bacillus* in the gut of mice (Tam et al. 2006) and the excretion of a higher dose of *Bacillus* spores in the faeces of pigs (Leser et al. 2008) or mice (Hoa et al. 2001) compared to the inoculum originally ingested by the animals. These findings indicate that the re-sporulation process in the gut and especially the large intestine plays a key role in completing the life cycle. It has been shown that the formation of endospores in *Bacillus* are promoted by conditions such as, a high cell density (Grossman and Losick 1988), nutrient limitation (Schaeffer et al. 1965), high mineral composition, or neutral pH that are in line with conditions found in the large intestine. There is however strain dependent differences in the response to these environmental cues (Bernlohr and Leitzmann 1969). Although sporulation of *B. subtilis* is induced by starvation, sporulation is not immediately initiated when growth slows due to nutrient limitation. Other alternative responses can occur, such as activation of flagellar motility to seek new food sources by chemotaxis, or secretion of hydrolytic enzymes to scavenge extracellular proteins and polysaccharides (González-Pastor 2012). Sporulation is the ultimate response to starvation and is thus suppressed until alternative responses are shown to be

inadequate. The sporulation in the intestine and exit from the host as an environment resistant spore completes the life cycle of the *Bacillus*.

Human and human GIT models

Data obtained from human studies has been treated separately from that of mammals, since the majority of data has been generated from artificial GIT models. Only one recent study has involved human volunteers. Although, the evidence from animals has shed light on the life cycle of *Bacillus*, the data from humans is more scarce. Assessment of *Bacillus* spore germination has been studied in vitro in a variety of human GIT models, from batch compartments (Ceuppens et al. 2012a) to complex dynamic interconnected systems like the Intestinal Models (TIM) developed by The Netherlands Organisation (TNO) for applied scientific research, (Hatanaka et al. 2012) and the Simulator of Human Intestinal Microbial Ecosystem (SHIME) from Prodigest (Ceuppens et al. 2012b) (Table 4). These models have been used to evaluate *Bacillus* from two different perspectives; on one hand, *B. cereus* pathogenic strains were assessed for the health risks (Ceuppens et al. 2010; Ceuppens et al. 2012a, b, c); and on the other hand, beneficial probiotic *Bacillus* candidates were applied in the models to assess the beneficial effects in human nutrition (Duc Le et al. 2004a; Hatanaka et al. 2012; Maathuis et al. 2010). These models are interesting since they allow you to determine phase when and where in the gut, spores germinate. Based on the studies above, it may be concluded that in most human GIT models, germination may be detected. However, the number of vegetative cells detected varies depending on specific gut sections. Bile appears to be major limiting factor, since an early germination of the spores in the upper part may reduce the final proportion of vegetative cells reaching the ileum and colon (Ceuppens

Table 3 Overview of studies of germination and overgrowth of sporulated *Bacillus* fed to pigs and broilers

Animal Target	Strain	Dose/Physiological status	Germination (G) or not	Results	References
Pigs	<i>B. subtilis</i> BN		G	Germinate and multiply in GI tract	Ozawa et al. (1981)
Piglet stomach	<i>B. cereus</i> var toyoi Toyocerin		G	Rapid germination in stomach of piglets, cells undergo repeated germination and sporulation during intestinal passages	Jadamus et al. (2001)
Piglets	<i>B. subtilis</i> CH201 <i>B. licheniformis</i> CH200	1.28×10^8 SP/g feed for 14 days (1:1)	G	Stability of SP all along the GIT (gut samples) Both strains germinate in the GIT, particularly in the stomach, but only limited outgrowth of the VC occurs	Leser et al. (2008)
Grower finisher pigs	<i>B. subtilis</i> CH201 <i>B. licheniformis</i> CH200	1.6×10^8 SP/g feed from 60 to 120 days and then no supplementation for 7 days	G	Excretion of SP with linear regression, persistence of SP for 10 to 11 days after withdrawal of the supplementation Evidence of germination (x 2.14) but no outgrowth	Leser et al. (2008)
Pigs 65–70 kg	<i>B. subtilis</i> CH201 <i>B. licheniformis</i> CH200	SP added intragastrically in a dialyzed membrane and followed for 4, 6, 8 and 24 h	G	% of SP decreases and the % of VC increases The duodenum and the colon are more convenient for germination Germination occurs in stomach but VC are quickly killed Resporulation occurs in intestine No colonization	Leser et al. (2008)
Day-of-hatch, off-sex broiler chickens	<i>B. subtilis</i> PHL-NP122	Adminsitrated either as a single gavage dose or constantly in the feed SP group: 10^6 spores per gram of feed or gavaged with a single dose of 10^6 spores suspended in 0.25 mL of PBS per chick using a sterile ball-ended gavage needle	G	Continuous administration: the number of recovered spores was constant through 120 h in each of the enteric regions from chickens receiving spores supplemented in the feed. However, the number of recovered <i>B. subtilis</i> spores was consistently about 10^5 spores per gram of digesta, which is about a 1-log ₁₀ reduction of the feed inclusion rate, suggesting approximately a 90% germination rate in the GIT when fed Persistence: recovered <i>B. subtilis</i> spores decreased with time, with only approximately 10^2 spores per gram of sample by 120 h	Latorre et al. (2014)
Chicken crops	<i>B. cereus</i> var toyoi Toyocerin		G	Rapid germination in chicken crops	Jadamus et al. (2001)
1 day-old chick	<i>B. subtilis</i> SC2362 <i>rrnO-lac</i> + <i>cat</i> genes	1×10^9 SP/chick	G	Number of VC was found to surpass the number of SP after 20 h postdosing in all regions of the GI tract of chick → no answer whether it is germination +/– replication in the gut? VCs were found in all regions of the GIT SP are transient members of the GIT of chick and do not colonize Fast germination of SP: 20 h after SP doses were administrated	Cartman et al. (2008)

Table 3 continued

Animal Target	Strain	Dose/Physiological status	Germination (G) or not	Results	References
Chicken	<i>B. thuringiensis</i> subsp. <i>kurstaki</i> transformed Bt HD-73GFP	10 ml VC 10 ml SP	G?	Bt harbors the marker gene <i>gfp</i> and erythromycin resistant Bt was found for the first 4 days after being fed VC of Bt Bt was found from day 1 to 13 in chicken feces of group fed with SP Bt	Zhang et al. (2012)

VC vegetative cell, G germination, SP spore, GIT gastrointestinal tract, P persistence, RT-PCR reverse transcription polymerase chain reaction

et al. 2012b). Hatanaka et al. (2012) demonstrated that only 8% of the spores germinated after passage through the upper part of the GIT model, but it was enough to influence a shift in the colon microbiota and exercise a probiotic effect. Dose, strain, experimental design, inter-experimental variation and how the *Bacillus* cultures were prepared may also explain varying results. Thus, the use of Human GIT models suggests that the life cycle of *Bacillus* spores is comparable to that of mice and pigs. These in vitro data have recently been supported by a clinical study (Ghelardi et al. 2015); four strains of *B. clausii* were orally administered to healthy adult volunteers and the fate of the spores was investigated. The authors used specific antibiotic resistance profiles of each of the probiotic *Bacillus* strains to detect them in faeces. The study demonstrated that the strains not only survived in vivo GIT conditions, but also underwent germination, outgrowth and multiplication as vegetative forms.

In conclusion, whatever the model and physiological characteristics, there is evidence of possible germination of *Bacillus* spores in the GIT especially in the stomach and small intestine. The rapid germination just after ingestion in the stomach is not the ideal pathway due to the presence of gastric juices and bile, however, germination in the first part of the small intestine which is nutrient rich and possesses a low microbial pressure would provide a good environment for reproduction. Furthermore, the sensitivity of vegetative cells to this stress is highly dependent on the growth status. Vegetative cells in the exponential phase are more sensitive to gastric and duodenal conditions compared to vegetative cells in the stationary phase. Conversely, a late germination in the large intestine reduces the opportunities of outgrowth due to: (1) the increased sensitivity of vegetative cells in the exponential phase and (2) the shorter transit time. Also, *Bacillus* strains and the dose administered appear to be crucial determinants that impact the entire life cycle in the GIT.

It seems clear that *Bacillus* spores can germinate in conditions present in the upper GIT, but it is also obvious that not all spores will germinate before excretion, particularly in species with a short transit time of 2 to 4 h such

as shrimp (Beseres et al. 2006). Also, germinated *Bacillus* may sporulate in the small intestine and in essence a mixture of spores and vegetative cells may be found in the intestine. Ingested spores of *Bacillus* transit through the GIT according to a ratio of vegetative cells to spores which may vary in different gut sections according to transit time, gut motility, microbiota, environmental conditions (e.g. pH, oxygen level, nutrient availability) and the nature of the feed. These parameters have not been fully assessed in the probiotic efficacy studies with *Bacillus* and would be important to include in future studies in order to better understand the correlation between the efficacy and life cycle of the *Bacillus* and to align observed probiotic benefits in the host to a physiological *Bacillus* status. Therefore, we decided to review the probiotic mechanisms associated with *Bacillus* and the potential differences between spores and vegetative cells in regards to probiotic benefits.

Probiotic efficacy of spores and vegetative cells

Bioactive secondary metabolites

Bioactive microbial secondary metabolites are components which regulate growth processes, replications, and/or exhibit responding (regulating, inhibiting, stimulating) action to the metabolism of cells at the biochemical level, in minimal concentration and *Bacillus* strains are well-known to produce secondary metabolites (Sansinenea and Ortiz 2011). These metabolites are exclusively produced by vegetative cells and generally at the highest levels during the transition from active growth to the stationary phase and thus not by the dormant spores.

An important category of secondary metabolites for the probiotic function of the *Bacillus* is the production of antimicrobial compounds, which is also commercially utilized. About half of the bacterial biocontrol agents are prepared from *Bacillus* strains. The antimicrobials include well documented molecules like bacteriocins and bacteriocin-like inhibitory substances (e.g., subtilin and coagulins)

Table 4 Overview of studies of germination and overgrowth of sporulated *Bacillus* in human or human gastrointestinal tract models

Human or GIT Model	Strain	Dose Physiological status	Germination (G) or not	Results	Reference
Healthy adult volunteers	Mixture of 4 <i>B. clausii</i> strains Enterogermina®	2×10^9 SP/capsule or vial	G, outgrowth and multiplication	The unique human in vivo experiment demonstrating germination of spores	Ghelardi et al. (2015)
Simulated gastric fluid (SGF) and Simulated intestinal fluid (SIF)	<i>B. subtilis</i> PY79 <i>B. pumilus</i> Biosubtyl ^{NT} <i>B. clausii</i> Enterogermina® <i>B. cereus</i> var. vietnami Subtyl <i>B. cereus</i> Biosubtyl ^{DL} <i>B. cereus</i> Bactilsubtil	From 10^7 to 5×10^8 SP/ml	Poor Resistance	Sensitivity to SGF: Biosubtyl ^{NT} , Subtyl (0.02% survival after 1 h) and Bactilsubtil Sensitivity to SIF: Subtyl (0.2% survival after 3 h) and Bactilsubtil mainly to bile	Duc Le et al. (2004a)
TIM-1 Human (model for upper part: stomach + small intestine)	<i>B. coagulans</i> GBI-30 (GanedenBC ³⁰)	2×10^9 SP per compartment	No G	70.4% of SP survived after 6 h in TIM-1. Not able to determine the amount of VC due to overgrowth of endogenous microbiota. No effect of SP supplementation on protein digestion of the diet but improvement of lactose and fructose digestion. Supplementation of diet with lactose and fructose on top of the SP may facilitate quick germination of SP. However, quick germination may induce higher mortality of VC in upper part of the GIT	Maathuis et al. (2010)
SHIME model	<i>B. cereus</i> NHV 1230-88	5.7 log CFU/ml of VC 4.6 log/ml of SP	G	All VC were inactivated during gastrointestinal passage. <i>Bacillus</i> SP germinated and grew out during the gastrointestinal simulation experiment	Ceuppens et al. (2010)
TIM-1 and TIM-2	<i>B. subtilis</i> C-3102 Calsporin®	9×10^7 SP/g of meal in TIM-1 4×10^9 SP in TIM-2	G (8%)	In TIM-1 (stomach → ileum), survival of SP is 99 and 8% have germinated In TIM-2 (colon) → effluent from TIM-1 containing SP is more efficient in increasing <i>Bifidobacterium</i> and decreasing <i>Clostridium</i> compare to SP added directly to TIM-2 suggesting that oral passage is essential and germination even small may be required	Hatanaka et al. (2012)
GIT model of human SHIME	<i>B. cereus</i> NVH 1230-88 <i>B. cereus</i> 14579 + Ery [®] plasmid	10^7 Exp. VC 10^7 Stat. VC 10^7 SP	G in duodenum only	<i>Gastric part</i> Exponential VC dyed; Stationary VC less sensitive but dyed after 4 h; SP are not inactivated but do not germinate after 8 h <i>Duodenum</i> Major part of the exp. VC is inactivated but residual VC are rapidly able to grow; stat. VC can grow; SP germinate within 1 h <i>Ileum</i> (only DNA copy, no distinction between SP or VC): Inactivation of VC and stability of SP: no growth and no germination	Ceuppens et al. (2012b)
In vitro model for bile resistance test	<i>B. cereus</i> ATCC 14579 <i>B. cereus</i> NVH1230-88 <i>B. cereus</i> FF73	VC and SP	No G	Bacteriocidal effect of bile is pH dependant VC that resist to gastric passage are inactivated in the intestinal environment by bacteriocidal effect of bile SP are not inactivated by bile but do not germinate until [bile] $< 5.0 \text{ g l}^{-1}$ SP germination is bile concentration dependant	Ceuppens et al. (2012a)

Table 4 continued

Human or GIT Model	Strain	Dose Physiological status	Germination (G) or not	Results	Reference
In vitro model with 5 compartments	<i>B. cereus</i> RIVM 9903295-4 <i>B. cereus</i> NVH1230-88 <i>B. cereus</i> LFMFP 381 <i>B. cereus</i> LFMFP 710	1×10^7 SP/ml	G No outgrowth of VC	Survival and germination of SP Outgrowth of VC is suppressed by human intestinal bacteria	Ceuppens et al. (2012c)

VC vegetative cell, G germination, SP spore, GIT gastrointestinal tract, P persistence, Exp exponential Stat stationary

or antibiotics (e.g., surfactin, iturins A, C, D, E, and bacilysin) (Mondol et al. 2013). These bioactive secondary metabolites are often a key factor in the screening process of probiotic candidates for food production animals, since the prevention of microbial diarrhea is a key aspect of animal performance.

Vegetative *Bacillus* cells also have a capacity to secrete extracellular carbohydrate-, lipid-, and protein-degrading enzymes that digest food and feed (Schallmeyer et al. 2004). Although the efficacy of animal growth performance resulting from feed supplementation with *Bacillus* spores is inferior to that achieved with exogenous enzyme supplementation, *Bacillus* species are considered attractive sources of various extracellular hydrolytic enzymes that could aid in nutrient digestion and feed utilization. For example, feed supplementation with protease, amylase, and lipase secreting *Bacillus*, improved the growth performance of broiler chicks (Santoso et al. 2001). In shrimp, *Bacillus* added to feed resulted in increased specific activity of lipase, protease and amylase in the digestive tract (Ziaei-Nejad et al. 2006). These studies indicate that spores in feed germinate and become metabolically active in the GIT to secrete digestive enzymes. However, it can be difficult to distinguish between the activity of enzymes endogenous to shrimp and enzymes synthesized by *Bacillus* and it has been suggested that the presence of the probiotic strain could stimulate endogenous enzyme production by the shrimp.

Detoxification

Toxic compounds found in feed that may threaten animal health include heavy metals and mycotoxins. Extensive use of pesticides during crop production and exposure of the environment to industrial toxic waste results in the deposition of heavy metals in soil and water, and this practice can pose serious threats to crop production and animal health through contaminated feed. Due to their high surface

area per unit weight, *Bacillus* spores and vegetative cells can act as biological adsorbents for dissolved toxins and metals. For example, it has been demonstrated that mature spores of a marine *Bacillus* sp. strain SG1 can oxidize Mn (II) to MnO_2 , and that vegetative cells of the same strain reduce MnO_2 in low-oxygen conditions (Mondol et al. 2013). The binding of ions is closely linked to the characteristic structure of the cell surface and therefore spores and vegetative cells act differently. It appears that the teichoic acids linked to the head groups of membrane lipids (lipoteichoic acids, LTAs) contribute to the adsorption of ions onto the cell walls of vegetative *B. subtilis* (Moriwaki et al. 2013); however, the LTAs are mainly absent in the spore and thus they have a different binding characteristic.

Mycotoxins are toxic secondary metabolites produced by fungi (moulds) that are formed during the growth phase of crops, but they can also be produced during storage or processing, when humid conditions occur. A wide range of microorganisms, including bacteria, moulds and yeasts, have demonstrated the ability to biotransform mycotoxins. Such microbes act in the animal GIT before absorption of the mycotoxins by the host can occur. Enzyme production by *Bacillus* strains has also been associated with the reduction of mycotoxins (Karlovsky 2011), and in vitro studies have demonstrated the removal of deoxynivalenol (DON) after incubation with culture supernatants of *Bacillus licheniformis* and *B. subtilis* (Cheng et al. 2010). The degradation of zearalenone by supernatants of *B. subtilis* 168 and *Bacillus natto* CICC 24640 has also been shown (Tinyiro et al. 2011). Based on in vitro screening, Bagherzadeh Kasmani et al. (2012) isolated hundreds of strains of vegetative forms of *Bacillus* with aflatoxin-binding capacity. Their best candidate (in fact a *Brevibacillus* strain) was fed in vegetative form to quails receiving an aflatoxin contaminated diet, and was shown to restore performance, serum biochemistry, and immune responses in vivo. Overall, these data indicate that vegetative cells are the key for detoxification. Nevertheless,

most of the *in vivo* studies on detoxification have not assessed the *Bacillus* forms in the GIT. These studies showed that *B. subtilis* was able to detoxify aflatoxin B1-contaminated feed and facilitate animal growth (Petchkongkaew et al. 2008) and that a preparation of *B. subtilis* ANSB060 had a protective effect on growth performance and meat quality of broilers, and reduced aflatoxin residues in the liver of broilers that were fed naturally moldy peanut meal (Fan et al. 2013). A *Bacillus* strain isolated from fish intestines was successfully used to detoxify deoxynivalenol in moldy corn that was fed to pigs (Li et al. 2011). On the other hand, a commercial product containing spores of *B. licheniformis* CH200 and *B. subtilis* CH201 at a density of 2.3×10^6 colony-forming units per g diet, failed to prevent absorption and toxic effects of the *Fusarium* toxin DON in piglets, suggesting that the probiotic bacteria neither bound nor degraded DON prior to absorption (Dänicke and Döll 2010) and that detoxification may depend on the strain of *Bacillus*. Although the majority of the *in vivo* studies did not assess the physiological status of the *Bacillus*, based on the studies reviewed above, it is likely that both vegetative cells and spores were present in the GIT and that vegetative cells are responsible for the efficacy. Without further data, it is difficult to conclude if spores in the GIT have a role in detoxification or what could be their contribution as absorbents.

Competition for ecological niches

Beneficial, non-pathogenic, and mature microbial cultures can be introduced into the intestinal tract of an animal, to create an environment where the beneficial microbiota outcompete opportunistic pathogens and reduce the chance of pathogen colonization. Mechanisms widely recognized for probiotic strains are competition for nutrients and intestinal space, and adherence to intestinal surfaces that can reduce pathogen colonization. Adhesion properties are general traits of probiotic *Bacillus* strains, and it has been reported that *B. indicus* HU36, *B. subtilis* var. Natto, *B. subtilis* PY79, *B. subtilis* BS3 and *B. licheniformis* BL31 all adhere to a human epithelial colorectal adenocarcinoma cells model—Caco-2 cells (Jung et al. 2012). The capacity of adhesion, on the other hand, seems to be a strain specific property and dependent on the form of *Bacillus*. Spores and vegetative cells have been shown to attach *in vitro* to Caco-2 cells (Sánchez et al. 2009). *In vivo*, both vegetative cells and spores of *B. polyfermenticus* CJ6 could adhere and colonize the small and large intestines of mice (Jung et al. 2012). The different cell surface compositions of vegetative cells and spores may interfere with the capability of the strain to reside temporarily in the intestinal tract. Harimawan et al. (2013) studied the adhesion of *B. subtilis* spores and vegetative cells onto metal surfaces, as it

indicates biofilm formation. Their data showed that compared to vegetative cells, spores had greater adhesion on stainless steel. Although the surfaces of spores and vegetative cells were hydrophilic, the former displayed higher hydrophobicity, which may arise from a significant amount of proteins on the spore's surface. The higher hydrophobicity and the lower energy barrier of spores results in significantly higher adhesion forces, compared to vegetative cells (Harimawan et al. 2013). Bacteria at different growth stages are known to have different local surface heterogeneity properties, such as surface charge density, electrophoretic mobility, and hydrophobicity. Spores contain significantly more proteins and appendages on their surface (e.g. proteinaceous coat, exosporium) compared to vegetative cells (Sirec et al. 2014). It seems that both vegetative cells and spores may have adhesion abilities, but at different levels, thus the probiotic mechanism may be dependant on the life cycle of the *Bacillus* strain in the intestine.

Immune effects of spores and vegetative cells

Bacillus genus is also used as probiotics in animal nutrition to promote immunity against various commercially relevant pathogens. *In vivo* studies revealed that *Bacillus* can beneficially promote markers of immunity in both chickens and pigs (Scharek-Tedin et al. 2013; Schierack et al. 2007), but association with improved animal performance has not always been established (Lee et al. 2010, 2014). Only few studies have assessed the differences between vegetative cells and spores in generating immune responses and the experiments have been focused mostly on the functions of dendritic cells (DCs) and macrophages that are capable of phagocytosis.

Intestinal macrophages are important for maintaining homeostasis in the intestine by phagocytosis and killing of bacteria. In a murine study, *Bacillus* spores were shown to survive inside the macrophages for a longer time than the vegetative cells (Duc le et al. 2004b) and, interestingly, spores could germinate inside the cells before they were released (Huang et al. 2008). This leads to a longer retention time in the macrophages (Huang et al. 2008) and later activation of some cellular effector pathways. The authors showed that the vegetative cells upregulated the expression of Toll-like receptor (TLR) 2 and TLR4 in macrophages after 4 h of incubation, whereas it took 6 h with spores (Huang et al. 2008). Non-viable spores could not upregulate TLR's, which suggests that it is the vegetative cell that has the greatest influence on macrophage function (Huang et al. 2008). Taken together, it seems that the immune response is delayed with spores, and is different to vegetative cells.

The prototypical function of DCs is to guide the type of immune response by phagocytosing bacteria, travelling to lymphoid tissues, and presenting antigens to naïve T-cells. In vitro, *B. subtilis* has been shown to drive the maturation of avian bone marrow derived DCs and induce antigen presentation (Liang et al. 2013). In addition, *B. subtilis* has been found in Peyer's Patches, mesenteric lymph nodes and the spleen of mice (Duc le et al. 2003a), showing that Bacilli also induce the adaptive immune response. Although both spores and vegetative cells are carried by DCs, the form of *Bacillus* seems to influence the type of immune response, T helper cell 1 (Th1) or 2 (Th2) dominated, that is generated by the DCs. It was elegantly shown that *Clostridium tetani* antigens on a spore coat, induced Th1 bias, yet expression on a germinating spore (vegetative cells) induced Th2 bias of the immune response (Uyen et al. 2007). The authors suggested that longer persistence time of spores inside the phagocytes favors intracellular (Th1) responses (Uyen et al. 2007). In addition, it has been shown that non-viable (unable to germinate) *B. subtilis* spores can enhance expression of Th1 type cytokines in mice DCs and confer protection against H5N1 avian influenza (Song et al. 2012).

Although in experimental settings spores may favor Th1 and vegetative cells Th2 responses, in vivo, a mix of vegetative cells and spores is present as described above, and it may be more difficult to observe a clear-cut bias in the immune response. One study showed that orally administered *B. subtilis* spores to mice induced systemic IgG and secretory IgA with Th1 bias (Duc le et al. 2003b). Similarly, it has been found that spore or spore coat induce Th1 bias and production of interferon- γ (IFN- γ) (Duc le et al. 2003b). On the other hand, live and heatkilled spores have been shown to induce both Th1 and Th2 cytokines in Th1 prone C57BL6 mice and in Th2 prone BALB/c mice (de Souza et al. 2014). Also, Barnes et al. (2007) showed that feeding of mice with killed spores resulted in a balanced Th1/Th2 response. These studies above suggest that in vivo, the resulting immune response is dependent on multiple factors, including the host and the strain. Overall, it seems that *Bacillus* probiotics can have a wide effect on immune response that have features of both Th1 and Th2 responses.

Conclusion

In general, there is clear evidence that *Bacillus* spores can germinate in GIT conditions. Both the spores and the vegetative cells exhibit functional properties in conjunction with each other in the GIT, and this functionality seems to depend on the ratio between the spores and the vegetative forms. From a probiotic perspective, vegetative cells and spores have separate, but complementary functions. Some

properties like immunomodulation and adsorption can be promoted by both VCs and SPs, while others, like antimicrobial activity, are a unique and specific trait of vegetative cells, as summarized in Fig. 1.

The germination process that guarantees metabolic activity (e.g. secretion of antimicrobial compounds, enzymes, synthesis of SCFAs and competition for essential nutrients) is highly dependent on the strain itself, GIT location, environmental conditions, age of the animal and intestinal maturity, as well as the conditions applied during the sporulation process prior to ingestion. Only a few studies have demonstrated spore germination of *Bacillus* spp. from in vitro and in vivo approaches and more studies are warranted. However, the available studies involve, studying the germination process of spores using non-specific and non-accurate culture methods, that may fail to detect specific strains in complex environments of the GIT or on the other hand, highly performant molecular tools that use GMO or toxigenic strains. Thus, even though germination seems to be obvious, it is difficult to establish the ratio between spores and vegetative cells that influence their probiotic properties.

Since spores must germinate to exert most of their beneficial effects, it would be interesting to identify compounds that effectively stimulate spore germination in the gut. The need for effective decontamination regimens for spores of organisms such as *Clostridium difficile* and *B. anthracis*, has renewed interest in this area, as stimulating spore germination would be the first step towards increasing vulnerability to elimination (Setlow 2014). It has been demonstrated previously that the sporulation of *Bacillus* strains may influence the germination potential of *Bacillus* spores. *B. subtilis* spores prepared under different conditions have been shown to exhibit different germination kinetics with nutrient germinants (Wells-Bennik et al. 2016). Temperature during sporulation and composition of the sporulation media has been shown to effect the relative composition of the spore, especially DPA and calcium (Baweja et al. 2008). In vitro, the same authors have demonstrated that early sporulation at an alkaline pH can generate spores that were found to be more resistant to both acidic and basic pH conditions, which may help the survival of a higher percentage of viable spores in such a microenvironment. Furthermore, technology and specific conditions applied during sporulation appear to modify the nature of the coat protein cross-linking (Abhyankar et al. 2016) that may have implications on the adhesion capability of the spores to specific gut ecological niches, as they have been shown to be proteinaceous in nature (Fig. 1). It is likely that the manufacturing process, as well as stresses that probiotic *Bacillus* spores undergo during feed processing and storage, can directly influence spore survival, germination, and outgrowth in the gut as highlighted for

Fig. 1 Overview of specific and non-specific beneficial mechanisms of probiotic *Bacillus* attributable to viable vegetative cells and spores in the gastrointestinal of animals and associated metabolites or cell wall components (black arrow) when expected to occur (LTA: lipoteichoic acids)

food (Warda et al. 2015). Until now, little has been done in this research area, but the increasingly growing importance of the spore-surface display, which is a powerful technology that can effectively express bioactive molecules on the surface of *Bacillus* spores, should help to better understand the factors influencing sporulation (Wang et al. 2017). It is suggested that more investigation is done on how the technical sporulation step influences the spore behavior in vivo in the gut and how it affects the overall modes of action and benefits of the probiotic strains.

Finally, little research has been done in vivo in pigs and broilers or in animal in vitro GIT models, which would be necessary in order to develop accurate methods to trace *Bacillus* spores and vegetative cells along the GIT, to better understand the influence of vegetative cell and spore ratio on beneficial effects along the GIT. The inter-strain variation in sporulation, and germination properties, make the prediction and control of spore behavior challenging as recently demonstrated for 17 strains of *B. subtilis* including nine isolates from the same origin (Krawczyk et al. 2017). Further research is needed to clearly understand and monitor the life cycle of probiotic *Bacillus* at strain level

that would help in screening and developing new probiotic products with improved benefits for animal.

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