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REVIEW ARTICLE

Bacillus probiotics: an alternative to antibiotics for livestock production

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Summary

The use of probiotics as feed supplements in animal production has increased considerably over the last decade, particularly since the ban on antibiotic growth promoters in the livestock sector. Several *Bacillus* sp. are attractive for use as probiotic supplements in animal feed due to their ability to produce spores. Their heat stability and ability to survive the low pH of the gastric barrier represent an advantage over other probiotic micro-organisms. This review discusses important characteristics required for selection of *Bacillus* probiotic strains and summarizes the beneficial effect of *Bacillus*-based feed additives on animal production. Although the mechanism of action of *Bacillus* probiotics has not been fully elucidated, they are effective in improving the growth, survival and health status of terrestrial and aquatic livestock. *Bacillus* strains also have utility in bioremediation and can reduce nitrogenous waste, thereby improving environmental conditions and water quality. Finally, recent innovative approaches for using *Bacillus* spores in various applications are discussed.

Introduction

Livestock production plays important roles in developing countries. Besides providing food, livestock production is a significant source of income for family farms and contributes towards the economic growth of many countries. Since the 1960s, global livestock production has increased substantially, a direct consequence of the growing world population and the increased demand for food. The large-scale addition of antibiotic growth promoters (AGPs) to animal feed has contributed to the increase in livestock production. However, global public health concerns have been raised regarding AGPs and their role in the emergence of multidrug-resistant micro-organisms. The over-use of AGPs has resulted in the development of antibiotic resistance in animal microbial populations with the potential for transfer of antibiotic resistance genes from animal to human microbiota. Due to this concern, the use of antibiotics in animal feeds has been prohibited in many countries, with Sweden being the first to ban AGPs in 1986 (Castanon 2007). Denmark subsequently banned the use of AGPs in 1998 and was followed by the European Union which introduced a total ban on AGPs in 2006 (Castanon 2007). The prohibition on the sub-therapeutic use of antibiotics in animal feed resulted in decreased animal production (Cheng *et al.* 2014) due to higher rates of infections in livestock and has also increased the risk of food-borne infections in consumers (Hao *et al.* 2014). In order to overcome the problems associated with the ban of AGPs on livestock production, a number of replacements/alternatives have been proposed (Cheng *et al.* 2014). One such strategy that has proven effective is the use of probiotics.

Many strains of some *Bacillus* sp. are currently used as probiotic dietary supplements in animal feeds. *Bacillus* is a genus of Gram-positive, aerobic or facultative anaerobic, endospore-forming bacteria. The ability to form spores is beneficial and allows for long-term storage without the loss of viability compared to those containing nonspore-forming bacterium. Also, spores are able to survive the harsh, low pH of the gastric barrier and can reach the small intestine to exert their probiotic

properties (Cutting 2011). At the time of preparing this manuscript, more than 100 species and subspecies of the genus *Bacillus* have been reported (http://www.bacterio.net/b/bacillus.html). Out of all known *Bacillus* sp. only a few are commonly used as probiotics in humans and animals, these include *B. coagulans*, *B. clausii*, *B. cereus*, *B. subtilis* and *B. licheniformis* (Cutting 2011; Fijan 2014).

The use of probiotics in livestock feeds has increased considerably in the last decade, as they are principally associated with reducing disease and improving animal performance. However, the majority of the currently used probiotics are based on lactic acid bacteria, mainly *Enterococcus* sp. and *Lactobacillus* sp. This review describes the state of the art of *Bacillus* probiotic research for use with terrestrial and aquatic livestock, with a critical evaluation of the screening of *Bacillus* species as potential probiotics and their effects on animals. Moreover, current technological applications for *Bacillus* spores are discussed.

Sources and general criteria for selection of ideal *Bacillus* probiotics

Potential *Bacillus* probiotics used in animals are typically isolated from the gastrointestinal tracts (GITs) and faeces of different animal species including chickens, pigs, ruminants and aquatic animals. *Bacillus* probiotics are often isolated from the animal's own GIT; however, cross-species use of isolated *Bacillus* strains is not uncommon. *Bacillus* sp. are saprophytes commonly associated with soil, water, dust and air. These bacteria are normally allochthonous to the GITs and are found as a result of ingestion of bacteria associated with soil and contaminated food (Hong *et al.* 2005). In addition to an intestinal origin, *Bacillus* probiotics can also be isolated from other sources, including food, plants, marine algae, aquatic habitats and soil. *Bacillus* probiotics isolated from different sources are summarized in Table 1. Most probiotic strains belong to *B. subtilis* or closely related species and were identified based on 16S rRNA gene sequencing, biochemical tests or multilocus sequence analysis. These *Bacillus* probiotics have been used in animal models of either terrestrial (i.e. chicken, pig, cow) or aquatic animals (i.e. crab, shrimp, sea cucumber). The detailed information on the use of each strain in animal models is shown in Table 1.

Functional criteria for desirable probiotic properties

To exhibit beneficial effects on the host, probiotic bacteria must be able to survive, colonize and persist, at least transiently, in the GIT. *Bacillus* sp. exists in both vegetative and spore forms. Vegetative cells are reported to be very susceptible to gastric acid and bile salts, while spores are resistant to both conditions (Barbosa *et al.* 2005). However, this is not always the case for *Bacillus* sp., Duc *et al.* (2004) have shown that spores of some commercial probiotic *B. cereus* strains were extremely sensitive to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The loss of spore viability after exposure to SGF and SIF was due to low pH-induced spore germination (as opposed to heat-induced germination). Although *Bacillus* sp. present in commercial products are usually consumed as spores, an assessment of the survival rate of vegetative cells exposed to simulated GIT conditions may be necessary. Several studies have shown that *Bacillus* spores can germinate in the small intestine (Cartman *et al.* 2008), and may exert their beneficial effect in the animal hosts through metabolically active mechanisms, such as secretion of antimicrobial substances and/or competition with pathogenic bacteria for essential nutrients (Duc *et al.* 2004).

The ability to adhere to intestinal epithelial cells is a virulence factor in the case of true pathogens; however, it is also an important beneficial property of potential probiotic strains. In general, the spores of *Bacillus* are more hydrophobic and show greater adhesion compared to the corresponding vegetative cells (Harimawan *et al.* 2013), although a few exceptions have been noted (Sánchez *et al.* 2009). A correlation between surface hydrophobicity and adherence of *Bacillus* strains has also been reported (Thwaite *et al.* 2009). The high hydrophobicity of spores is probably due to the presence of a proteinaceous coat and exosporium on their surface (Harimawan *et al.* 2013). However, it is not clear whether the exosporium or other spore components play a major role in adhesion to host cells. Since vegetative cells lack such structures adhesion cannot be explained solely by hydrophobicity. Among the surface-associated proteins from vegetative cells and spores of probiotic *B. cereus* identified by Sánchez *et al.* (2009) several have been found to be important for adhesion. Surface-associated proteins from *Bacillus* including S-layer proteins, an aminopeptidase, a flagellin and a cell envelope-bound metalloprotease are specifically bound to mucin and fibronectin and might play important roles in the adhesion of this probiotic strain to the GIT (Sánchez *et al.* 2009).

Bacterial attachment to intestinal epithelial cells is not only beneficial for their colonization in the gut but can also stimulate immune cells of the gut-associated lymphoid tissue. There is strong evidence that *B. subtilis* spores can translocate across microfold (M) cells and move into the Peyer's patches before being transported to efferent lymph nodes (Duc *et al.* 2003). Peyer's patches are rich in antigen-presenting cells (dendritic cells,

Table 1 Sources of *Bacillus* isolation

Sources	*Bacillus* strains	Identification techniques	Animal model(s)	References
Intestinal				
Fish gut	*B. subtilis* ANSB060	Standard morphological, biochemical, physiological tests and 16S rRNA gene sequencing	Broiler	Fan *et al.* (2015)
Healthy chicken gut	*B. subtilis* PB6 (ATCC-PTA 6737)	Biochemical test and 16S rRNA gene sequencing	Chicken	Teo and Tan (2005); Jayaraman *et al.* (2013)
Sea cucumber intestine	*B. subtilis* T13	Cluster analysis on the sequences of 16S rRNA gene	Sea cucumber	Zhao *et al.* (2012)
Mud crab's intestinal tract	*B. pulmilus* BP *B. subtilis* DCU	16S rRNA gene sequencing	Mud crab	Wu *et al.* (2014)
Shrimp's digestive tract	*Bacillus* OJ	Standard morphological, biochemical and physiological tests	Shrimp	Li *et al.* (2009)
Faeces				
Healthy calf faeces	*B. coagulans*	Metabolic profiles and 16S rRNA gene sequencing	Calves	Ripamonti *et al.* (2009)
Food				
Fermented soybean natto	*B. subtilis* Natto	16S rRNA gene sequencing	Cow and chicken	Peng *et al.* (2012) Sun *et al.* (2013)
Soybean	*B. subtilis* LS1-2	Biochemical test and 16S rRNA gene sequencing	Broiler	Sen *et al.* (2012)
Fermented pickles	*B. subtilis* strains, L10 and G1	Biochemical test and 16S rRNA gene sequencing	Shrimp	Zokaeifar *et al.* (2014)
Soybean mash	*B. subtilis* DSM 5750	Multilocus sequence analysis and pulsed field gel electrophoresis	Piglet, pig, calves and young lamb	Alexopoulos *et al.* (2004); Kritas *et al.* (2006); Kowalski *et al.* (2009); EFSA (2016a)
Soil	*B. subtilis* C-3102	Biochemical test, 16S rRNA gene sequencing and restriction pattern analysis	Chicken	EFSA (2015)
	B. subtilis CBS 117162	Standard morphological, biochemical and 16S rRNA gene sequencing	Piglet and pig	EFSA (2011)
	B. licheniformis DSM 5749	Partial gyrA and rpoB sequences analysis	Piglet, pig, calves and young lamb	Alexopoulos *et al.* (2004); Kritas *et al.* (2006); Kowalski *et al.* (2009); EFSA (2016a)
Other				
Shrimp ponds	*B. subtilis* UTM126	Standard morphological, biochemical and physiological tests	Shrimp	Balcázar and Rojas-Luna (2007)
Chinese herbs	*B. subtilis* MA139	Standard morphological, biochemical, physiological tests and 16S rRNA gene sequencing	Piglet	Guo *et al.* (2006)
Hay	*B. subtilis* DSM 28343	*gyrA* and 16S rRNA gene sequencing, and pulsed field gel electrophoresis	Chicken	EFSA (2016b)
Seaweed	*B. pumilus* WIT 588	*gyrB*, *pyrE* and 16S rRNA gene sequencing	Pig	Prieto *et al.* (2014)
Shrimp ponds	*B. licheniformis*	Biochemical, physiological tests and 16S rRNA gene sequencing	Shrimp	Li *et al.* (2007)
Hydrogen-producing fermented solution	*B. fusiformis*	16S rRNA gene sequencing	Shrimp larviculture	Guo *et al.* (2009)

macrophages) involved in processing and presenting antigen to B cells for production of secretory IgA (sIgA). Stimulation of the sIgA response is necessary for immunity against mucosal pathogens. Several studies have also shown that *Bacillus* spores can enhance the innate immune system and macrophage phagocytosis (Duc *et al.* 2004; Xu *et al.* 2012). Modulation of immune function or stimulation of host defence systems are required

properties of potential probiotics and are related to the antagonistic effects of probiotics.

Antagonistic activities of probiotic strains are essential to prevent or reduce infection with pathogenic bacteria. The production of antimicrobial compounds is often associated with antagonistic activities, and this is the first functional property considered when selecting potential probiotics. Members of the genus *Bacillus* are known to produce a number of antimicrobial compounds, including lipopeptides, surfactin, bacteriocins and bacteriocin-like inhibitory substances. These antimicrobial agents are typically active against Gram-positive bacterial pathogens, but some display activity against Gram-negative bacterial pathogens as well as fungal pathogens (Kerr 1999; Teo and Tan 2005; Khochamit *et al.* 2015). The antagonistic activities of probiotics also include the production of organic acids that lower pH and competitive exclusion (CE) of pathogens. The CE is defined as the ability of the beneficial micro-organism to compete with potentially harmful bacteria in terms of adhesion sites. An example is the ability of *B. subtilis* to inhibit the adhesion of *Salmonella* Enteritidis and enterotoxic *Escherichia coli* to the surface of intestinal epithelial cells (Thirabunyanon and Thongwittaya 2012; Ye *et al.* 2013).

In addition to these basic probiotic characteristics, *Bacillus* sp. also have additional functional properties that could promote animal health and welfare. Several *Bacillus* sp. are able to produce a variety of extracellular enzymes including amylase, protease, lipase, phytase, cellulase and xylanase (Latorre *et al.* 2016). These enzymes have been used in animal nutrition to assist in the digestion of feed and improve nutrient absorption. *Bacillus* strains also exhibit bile salt hydrolase activity, cholesterol-reducing ability and antioxidant activity (Shobharani and Halami 2016) that may exert beneficial health effects by lowering the serum cholesterol levels and relieving oxidative stress.

Safety criteria for selection of probiotic strain

Evaluation of the safety of a probiotic begins with the correct identification of the strains. The taxonomic identification of a micro-organism based on the integration of phenotypic and genotypic properties is imperative in differentiating it from its pathogenic relatives or other harmful micro-organisms. Phenotypic identification of probiotic bacteria using classic microbiological approaches is important for species identification but is not always reliable and certain species cannot be distinguished by these methods alone. Identification of *Bacillus* strains at the species-level must be confirmed using molecular methods. Species-level identification often relies on analysis of the 16S ribosomal DNA (16S rDNA) sequence. However, the 16S rDNA sequence alone provides insufficient resolution to differentiate between closely related *Bacillus* species. For example, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis* and *B. pumilus* cannot be uniquely identified using 16S rDNA alone. Analysis of *gyrA* or *gyrB* genes may be needed in addition to the 16S rDNA sequence (EFSA 2014).

The capacity for toxin production associated with *Bacillus* (EFSA 2014) is a principal safety concern for consumption by humans as well as livestock. *Bacillus cereus* is the most important cause of food poisoning, with diarrhoeal and the emetic syndromes resulting from toxin production. The diarrhoeal type of food-borne illness is caused by the pore-forming cytotoxins haemolysin BL (Hbl, three-component toxin encoded by hbl*CDA*) and nonhaemolytic enterotoxin (Nhe, three-component toxin encoded by nhe*ABC*). Whereas, the emetic type of food-borne illness is due to the production of cereulide, a heat-stable emetic toxin encoded by the *ces* gene. *Bacillus cereus* also harbours the capacity to produce many other toxins such as enterotoxin T (BceT, encoded by *bceT* gene), enterotoxin FM (EntFM, encoded by *entFM* gene) and cytotoxin K (CytK, encoded by the *cytK* gene). In addition to the well-established toxins mentioned above, several additional virulence factors such as haemolysin A, haemolysin II, haemolysin III, phosphatidylinositol-specific phospholipase, cereolysin A (phospholipase C), cereolysin B (sphingomyelinase), cereolysin O and their pleiotropic transcriptional activator PlcR (Kim *et al.* 2015), as well as lecithinases (Duc *et al.* 2004) also contribute to the enterotoxic activity of *B. cereus* strains. In principle, strains belonging to the *B. cereus* taxonomic group are considered inadvisable for direct use in animal production (EFSA 2014); however, if they are proposed for use, then the full genome should be sequenced and analysed to search for genes that are responsible for the production of enterotoxins and the emetic toxin (EFSA 2014). If there is evidence of homology, the nonfunctionality of the genes (e.g. mutation, deletion) should be demonstrated (EFSA 2014).

Besides the well-known pathogenic *B. cereus*, *Bacillus* sp., other than members of *B. cereus* group, have occasionally been reported to be associated with food-borne illness. Several other *Bacillus* sp. were reported to produce substances toxic to mammalian cells, such as the heat-stable toxin amylosin from *B. subtilis, B. amyloliquefaeiens* and *B. mojavensis* which have been connected with food poisoning (Mikkola *et al.* 2007; Apetroaie-Constantin *et al.* 2009). Lichenysin A and Pumilacidin are found in *B. licheniformis* and *B. pumilus* strains, respectively, and are associated with incidences of food poisoning (Salkinoja-Salonen *et al.* 1999; From *et al.* 2007). Some strains of *B. megaterium*, *B. simplex* and *B. firmus* produce a heat-stable toxin, which shows similar physical

characteristic to the *B. cereus* emetic toxin, cereulide (Taylor *et al.* 2005). Accordingly, an *in vitro* cytotoxicity test using Vero cells or other epithelial cell lines is recommended for the assessment of non-*B. cereus* group, and strains which demonstrate cytotoxicity are not recommended for use (EFSA 2014).

Another concern that should be taken into consideration is the potential of some *Bacillus* strains to transfer antibiotic resistance genes within the GITs that might take place between probiotics and opportunistic or pathogenic bacteria. Genes conferring resistance to aminoglycosides (*aadD2*), macrolides (*erm34*), β-lactams (bla_{BCL-1}) and chloramphenicol (cat_{Bcl}) were found in probiotic strains of *B. clausii* (Bozdogan *et al.* 2003, 2004; Girlich *et al.* 2007; Galopin *et al.* 2009). β-lactams, chloramphenicol and tetracycline resistance determinants have also been reported in probiotic strains of *B. cereus* (Hoa *et al.* 2000). The tetracycline resistance gene, *tet* (M) of the *B. cereus* group was also found on the Tn*916*-like transposon and this mobile element could be transferred to *B. subtilis*, *S. aureus* and enterococci (Agersø *et al.* 2002). Other tetracycline resistance genes, such as *tet* (K), which confers chlortetracycline resistance in *B. subtilis*, was found on a plasmid and could be transferred from *B. subtilis* to *E. coli* by conjugation (Dai *et al.* 2012). Genes conferring resistance to erythromycin (*ermD* and *ermK*) are reported in *B. licheniformis*. Gryczan *et al.* (1984) observed that the *ermD* gene is located on the chromosome of *B. licheniformis*, contrary to the report by Adimpong *et al.* (2012), who found that the *ermD* and *ermK* genes are located on a plasmid.

Due to a serious concern for the development of antibiotic resistance and transference of antibiotic resistance genes among bacteria, the EFSA has suggested that products containing *Bacillus* strains (and/or other bacterial species) intended for use as feed additives must be examined for susceptibility to antimicrobials of human and veterinary importance. Antimicrobials required to be examined in *Bacillus* sp. are vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol. The MIC cut-off values of bactericidal and bacteriostatic antibiotics are recommended at a low concentration of 4–8 mg l^{-1}. Strain(s) with MICs above the cut-off values for one or more antimicrobials require further investigation to determine whether the strain(s) exhibit intrinsic or acquired resistance. Strains resistant due to the acquisition of exogenous resistance genes are unacceptable for use as animal feed additives (EFSA 2012).

Technological criteria to evaluate strain viability and stability

Technological criteria related to feed production and processing are also important for the selection of probiotics along with functional and safety aspects. Unlike the commonly used probiotic lactic acid bacteria, strains of the *Bacillus* sp. are usually considered to be very stable due to their ability to form endospores. Endospores are highly resistant to physicochemical stress during feed production and storage, such as high pellet temperature, pressure and shear forces. *Bacillus* spores are resistant to a pelleting temperature of up to 90°C with over 90% of spores remaining viable in feed samples (Amerah *et al.* 2013).

These criteria are important requirements for the selection of probiotic strains. However, the development of suitable probiotics is not a simple task and requires full-scale trials. *In vitro* analysis allows for preliminary screening of probiotic candidates and the results of these studies may help predict the *in vivo* effects. However, in some cases, results of *in vitro* studies have not been linked to *in vivo* effects. Therefore, *in vivo* studies are needed to demonstrate the beneficial effect of candidate strains on the animal hosts before being used as a probiotic. Finally, economical evaluation and approval by an authorized party should be performed before commercialization. A diagram illustrating the different parameters required for the selection of *Bacillus* strains as probiotics for animal use is shown in Fig. 1.

Mode of action and efficiency of *Bacillus* in terrestrial and aquatic animals

Poultry

Probiotics have been used widely in poultry production, particularly with newly hatched chicks that are born with a sterile gut. Nurmi and Rantala (1973) proposed supplementation with microbes to restore protective gut microbiota, to facilitate CE. To date, a number of studies have demonstrated the CE effect of *Bacillus* in reducing pathogen colonization in poultry. La Ragione and Woodward (2003) demonstrated that predosing newly hatched specific pathogen-free chicks with a suspension of 1×10^9 spores of *B. subtilis* $PY79^{hr}$ is sufficient to suppress colonization and persistence of *S.* Enteritidis or *Clostridium perfringens* upon subsequent challenge. The shedding of *S.* Enteritidis was also reduced significantly in the predosed birds for the 36-day duration of the experiment. In addition, Jeong and Kim (2014) observed that *B. subtilis* C-3102 reduces the number of *C. perfringens* and Enterobacteriaceae in the excreta. Similarly, Guyard-Nicodème *et al.* (2016) showed that shedding of *Campylobacter* is reduced when broilers were fed *B. subtilis* C-3102. Feeding with either *B. subtilis* DSM17299 (Knap *et al.* 2011) or *B. cereus* var. *toyoi* (Toyocerin) (Vilà *et al.* 2009) significantly reduced *Salmonella* colonization in broilers. Supplementation of *B. subtilis* PB6 to broilers also

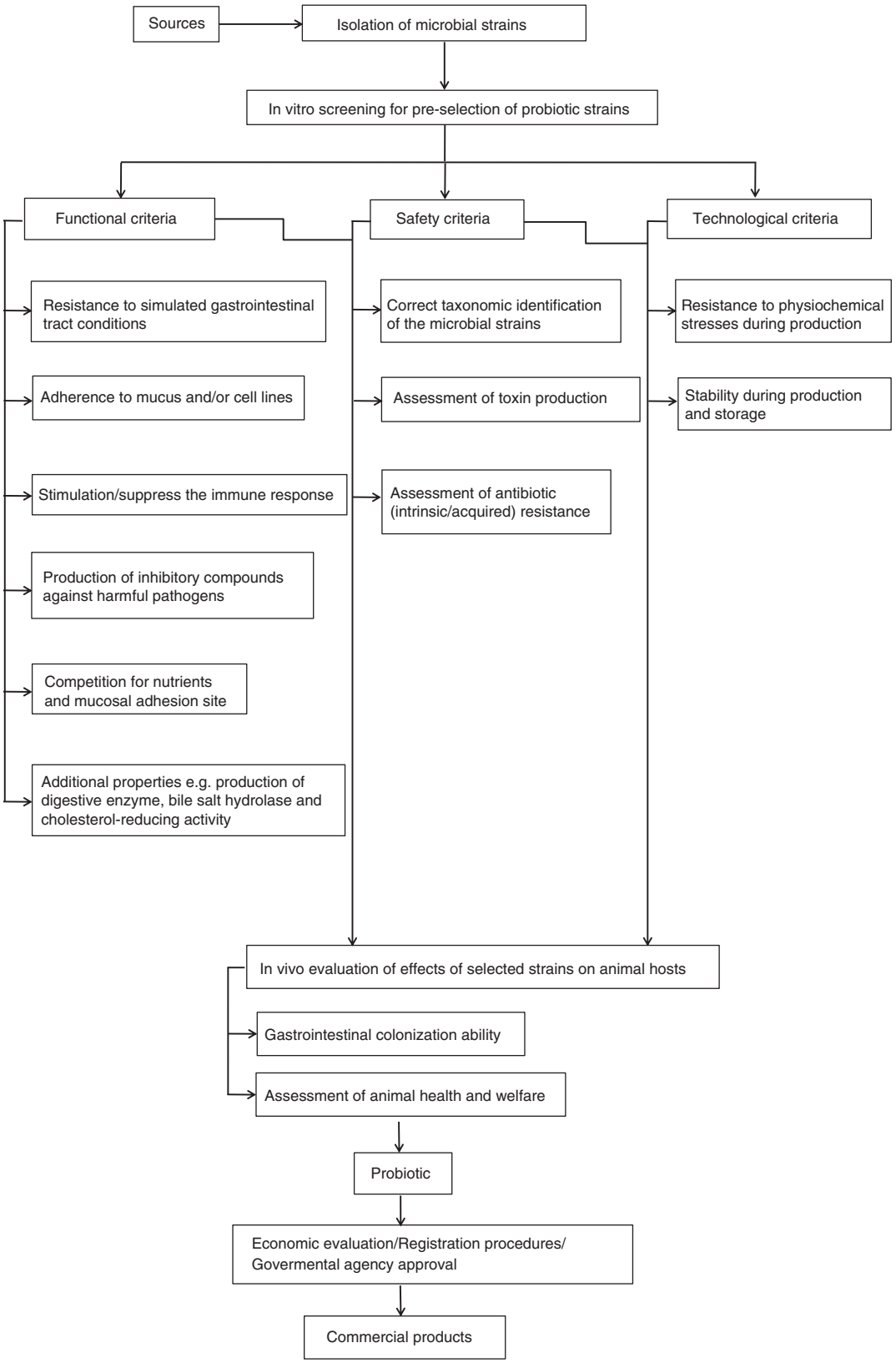

Figure 1 Diagram for selection of *Bacillus* as probiotics for animal use.

resulted in a reduction in intestinal *C. perfringens* counts (Jayaraman *et al.* 2013).

Besides suppressing the colonization of gut pathogens, feeding *Bacillus* probiotics to poultry can improve feed conversion and enhance weight gain. Sen *et al.* (2012) observed that administration of *Bacillus* causes histomorphological changes in the intestine of broilers, increasing villus height and villus height to crypt dept ratio, and thus improves the nutrient digestibility and absorption capacity of the small intestine. Broilers fed *Bacillus*-supplemented diets exhibit reduced digesta viscosity caused by soluble nonstarch polysaccharide, which can affect nutrient availability and absorption (Latorre *et al.* 2015). In addition to promoting the growth performance and health of poultry, feed with added *Bacillus* also improves the quality of meat (e.g. increases in the lightness, redness and yellowness of meat) (Yang *et al.* 2016) and eggs (e.g. increases in eggshell thickness, yolk colour, Haugh unit, and decreases in yolk cholesterol) (Xu *et al.* 2006). *Bacillus*-containing feed can also limit effects from toxin-contaminated feed in broilers (Fan *et al.* 2015) as well as reduce NH_3 emission from poultry manure (Jeong and Kim 2014).

Pigs

Similar to poultry, newborn pigs have a sterile gut and acquire their characteristic flora by contact with their mother and the environment. According to Kenny *et al.* (2011), the period immediately after birth and postweaning are the most vulnerable times in the life of the pig in which in-feed antimicrobials' withdrawal could affect mortality. During these periods, pigs are highly susceptible to gut colonization by pathogenic bacteria that are responsible for growth reduction and diarrhoea. In addition, weaning exposes piglets to biological stress that can contribute to intestinal and immune system dysfunctions, and may result in reduced pig health, growth and feed intake, particularly the first week postweaning. It has been claimed that the microbiota in the pig gut is unstable during the first week after weaning, and 2–3 weeks postweaning is required for gut microbes to reach a high level of stability and to fully develop their fermentative capacity (Jensen 1998). The piglet should, therefore, be exposed to a protective gut microbiota during these life stages which would protect against environmentally acquired pathogens by direct and indirect (stimulation of the host immune system) means, with the added benefit of improving nutrient digestibility. It has been well documented that *Bacillus* probiotics can serve as an alternative to AGPs for improving growth of piglets. Kyriakis *et al.* (1999) demonstrated that administration of *Bacillus* to weaned piglets reduced the incidence and severity of diarrhoea, as well as causing a significant improvement in feed conversion and weight gain. Furthermore, *Bacillus* can promote microbiota formation by stimulating the growth of beneficial bacteria. Hu *et al.* (2014) observed that supplementation of *B. subtilis* increased the relative number of *Lactobacillus* and also reduced *E. coli* levels, the most common cause of diarrhoea in weaned pigs.

In general, dietary supplementation with *Bacillus* direct-fed microbials gives more positive and consistent results in weaned piglets than in growing-finishing pigs. This is probably due to more stable gut microbiota, better digestibility and immunity, as well as increased resistance to intestinal disorders in older animals compared to weaned piglets. However, reports on the feeding of dietary *Bacillus*-based probiotics to growing-finishing pigs are rare and often contradictory. For example, when supplementing basal diets for growing-finishing pigs with BioPlus 2B (*B. licheniformis* and *B. subtilis*), Alexopoulos *et al.* (2004) observe an improvement in weight gain, feed conversion ratio and carcass quality, whereas Wang *et al.* (2009) and Jørgensen *et al.* (2016) did not observe positive effects on feed efficiency. The inconsistency for the effect of *Bacillus*-based probiotics on the performance of pigs may be ascribed to several factors, including diet compositions, the dose of *Bacillus* probiotic, the age of pigs and interaction with environmental factors.

In addition to the effect on the health status of pigs, *Bacillus* could indirectly lead to a reduction in environmental pollutants, such as faecal emissions from pig manure. Upadhaya *et al.* (2015) have proposed that the reduction in faecal NH_3 emissions from pigs supplemented with *Bacillus* was likely due to high nutrient digestibility. In contrast, Wang *et al.* (2009) did not observe an improvement in nutrient digestibility while slurry NH_3 emissions were reduced. These authors suggest that the decrease in NH_3 emission may be the result of alteration in the intestinal microbiota and a significant decline in the pH of the slurry.

Ruminants

In intensive farm systems, like monogastric animals, young preruminants such as calves, are separated from their mother before the microbial colonization of the gut is complete. In addition, neonatal calves are often stressed in new environments and by conditions such as transport, vaccination, weaning and dehorning. These situations have the potential to make calves more susceptible to infections that affect the GIT, and hence increase the risk of diarrhoea (also known as scouring) and weight loss, which is a major cause of morbidity and mortality during the early life of calves and other ruminants. Therefore, prevention of diarrhoea is important to

diminish mortality and promote the growth of calves. An investigation by Kritas *et al.* (2006) demonstrated that BioPlus 2B-treated young lambs tended to have lower diarrhoea mortality than the untreated control group. While Kowalski *et al.* (2009) found that rearing calves fed diets supplemented with BioPlus 2B showed improved feed intake and performance. Sun *et al.* (2010a) demonstrated that preweaning calves fed a *B. subtilis* natto showed increased general performance and improved average daily weight gain (ADG). In addition, *B. subtilis* natto supplementation also modulated the immune function and advanced the weaning age of the calves. However, contradictory effects of *Bacillus* administration on pre-ruminant calves have been reported. Riddell *et al.* (2010) did not observe a positive effect on growth performance and health parameters in young calves fed BioPlus 2B. Similarly, Jenny *et al.* (1991) also did not observe any differences in the same indices with the inclusion of a *Bacillus* probiotic. Riddell *et al.* (2010) indicated that probiotics are most effective during periods of stress. Thus, it is plausible that the lack of positive effect on performance, growth and health may be due to the lack of stress imposed on the calves in these studies, as the calves were housed indoors in a temperature-controlled environment with adequate ventilation. In addition, the switch from a milk base to a soy-based milk replacer may not have been sufficient to stress the calves to the point of dysbiosis.

The beneficial effects of the administration of *Bacillus*-based probiotics have been shown to extend to adult ruminants, although only limited information is available. In adult ruminants, probiotics have mostly been selected to improve fibre digestion by targeting the rumen compartment, which consists of a diverse array of strictly anaerobic bacteria and fungi, ciliate protozoa and archaea. These rumen micro-organisms are of great importance for fermentation and degradation of 70–75% of the dietary compounds (Chaucheyras-Durand and Durand 2010), as well as for the production of milk (Sun *et al.* 2013). Peng *et al.* (2012) and Sun *et al.* (2013) demonstrated that cows fed a *B. subtilis* natto fermentation product have higher feed efficiency, improved milk production and milk component yield, possibly due to alterations in the rumen fermentation pattern. Qiao *et al.* (2010) also investigated the effect of administration of 100 g day^{-1} (2×10^{11} cells) of live *B. licheniformis* to early lactation cows and observed increased milk yield and milk protein, as well as enhanced ruminal apparent nutrient digestibility of neutral detergent fibre, acid detergent fibre and organic matter. Another experiment regarding a *Bacillus*-based probiotic was conducted in milking ewes (Kritas *et al.* 2006), with milk yield, fat and protein content significantly increased after supplementation of BioPlus 2B at a dose of approximately $2{\cdot}56 \times 10^9$ viable spores day^{-1} ewe^{-1}.

Aquaculture

Unlike terrestrial farm animals that undergo embryonic development within an amnion, the larval forms of almost all aquatic animals are released into the external environment at an early ontogenetic stage. These larvae are highly exposed to gastrointestinal-associated disorders since they start feeding even though their digestive tract and the immune system is not fully developed. In addition, they constantly interact with opportunistic pathogens, which are a major cause of mortality and economic losses in aquaculture, through the processes of osmoregulation and feeding. Thus, probiotic treatments are required during the larval stages for optimal improvement of the indigenous gut microbiota. Guo *et al.* (2009) found that daily addition of *B. fusiformis* at a concentration of 1×10^5 CFU ml^{-1} can increase the survival and accelerate the metamorphosis of larval shrimp, *Penaeus monodon* and *Litopenaeus vannamei*. Similar findings were reported by Luis-Villaseñor *et al.* (2011). Another study performed with *Penaeus vannamei* larvae demonstrated an enhanced survival rate and increased activities of digestive enzymes (e.g. protease, amylase and lipase) after the addition of *B. coagulans* to the water (Zhou *et al.* 2009).

In aquaculture, probiotic treatments may also be considered as a form of biocontrol of pathogens. Numerous studies have examined the mechanisms by which *Bacillus* can control microbiota and confer resistance to disease. Vaseeharan and Ramasamy (2003) reported the addition of *B. subtilis* BT23 at a density of 10^6–10^8 CFU ml^{-1} in the cultivation of *P. monodon* prior to challenge with *Vibrio harveyi* at 10^3–10^4 CFU ml^{-1} showed a 90% reduction in accumulated mortality. This strain also showed inhibitory activity against *Vibrio* sp. using *in vitro* conditions. Balcázar and Rojas-Luna (2007) investigated the decrease in mortality of juvenile *L. vannamei* when the shrimp were fed a diet containing *B. subtilis* at 10^5 CFU g^{-1} for 28 days before a challenge with *V. harveyi* at 10^5 CFU ml^{-1}. They speculate that the mechanism by which *B. subtilis* prevents pathogen infection is based on CE of the pathogen, due to the presence of *B. subtilis* in the shrimp hepatopancreas at the completion of the study. A similar finding was reported by Boonthai *et al.* (2011).

The CE mechanisms by which *Bacillus* protects aquatic animals against pathogen infection can be extended to immunity enhancement. Li *et al.* (2007) found that *L. vannamei* fed *B. licheniformis* exhibited a significant increase in phenoloxidase and superoxide dismutase

activities, and showed a decrease in the population of *Vibrio* sp. in the intestine as compared with the control shrimp. Zokaeifar *et al.* (2012) also observed that *Bacillus* can activate immune defences in *L. vannamei*, which in turn contributed to improving the growth performance and survival of the shrimps. Similar phenomena were reported in other aquatic animals, such as juvenile sea cucumber, *Apostichopus japonicas* (Zhao *et al.* 2012), mud crab, *Scylla paramamosain* (Wu *et al.* 2014) and fish species (Sun *et al.* 2010b; Ai *et al.* 2011). In addition, some *Bacillus* probiotics exhibit antiviral activity against white spot syndrome virus in shrimp, although the precise mechanism is not known (Li *et al.* 2009).

In addition to biological control of disease, survival and growth of aquatic animals are also directly related to the quality of water. In many studies, water quality was recorded during the addition of probiotic strains, particularly *Bacillus* sp. Lalloo *et al.* (2007) reported a decrease in concentration of ammonium, nitrate and phosphate ions in aquaria treated with a mixture of equal proportions of three *Bacillus* isolates (1×10^8 CFU l^{-1}). Similar findings were observed by Zokaeifar *et al.* (2014).

Bacillus spore innovation: nanobiotechnology, surface display and adsorption for improving feed efficiency

Bacillus strains are not only useful as probiotics for farm animals but also have utility as a delivery vehicle for a variety of different molecules, in so-called nanobiotechnology. Efficient display systems have been development through construction of fusion genes containing the sequence of spore coat proteins. Numerous peptides or proteins have been fused to spore coat proteins and stably expressed on the spore surface. These peptides and proteins are involved in many diverse biological applications, including bioremediation, biocatalysts and vaccine development for protective immunization. For instance, proteins able to bind metal ions when expressed on the *Bacillus* spore coat can be used as bioadsorbents for the removal of heavy metals from contaminated ecosystems (Hinc *et al.* 2010). The display of enzymes able to degrade fibre or feed components on the surface of *Bacillus* spores can be used in animal feed to improve nutrient digestibility (Potot *et al.* 2010). Highly immunogenic proteins and peptides once exposed on the spore surface could be used to induce protection against pathogen infection in animals (Ning *et al.* 2011). Although protein display technology is an exciting development and has recently attracted considerable worldwide attention, concerns remain regarding the use of recombinant probiotic strains in humans and animals. In particular, the release of live genetically modified micro-organisms into the environment is a major concern for the use of all microbe-based display systems. To overcome this obstacle, a nonrecombinant display approach, which is based on the adsorption of heterologous proteins to the spore surface, without the need of genetic manipulations, has been recently developed. Several reports have shown efficient adsorption of protein antigens and enzymes (e.g. β-galactosidase, heat labile toxin) on the surface of *Bacillus* spores (Sirec *et al.* 2012; Isticato *et al.* 2013).

In conclusion, *Bacillus* sp. have been extensively studied and developed as commercial probiotic products for animal use. Recent studies have indicated that *Bacillus* probiotics positively contribute to the overall health status of their hosts. Future studies must investigate the mechanisms by which gut microbiota interact with host intestinal epithelium cells in order to define selection criteria for potential probiotics. The effectiveness of probiotics depends largely on the dose ingested and bacterial strains, therefore, it is essential to determine the minimal effective dosage of probiotic strains.

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Conflict of Interest

There is no conflict of interest.

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