



Bacillus Species as Direct-Fed Microbial Antibiotic Alternatives for Monogastric Production

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Abstract

Antibiotic growth promoters have been utilized for long time at subtherapeutic levels as feed supplements in monogastric animal rations. Because of their side-effects such as antibiotic resistance, reduction of beneficial bacteria in the gut, and dysbiosis, it is necessary to look for non-therapeutic alternatives. Probiotics play an important role as the key substitutes to antibacterial agents due to their many beneficial effects on the monogastric animal host. For instance, enhancement of the gut microbiota balance can contribute to improvement of feed utilization efficiency, nutrients absorption, growth rate, and economic profitability of livestock. Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.” They are available in diverse forms for use as feed supplements. Their utilization as feed additives assists in good digestion of feed ingredients and hence, making the nutrients available for promoting growth. Immunity can also be enhanced by supplementing probiotics to monogastrics diets. Moreover, probiotics can help in improving major meat quality traits and countering a variety of monogastric animals infectious diseases. A proper selection of the probiotic strains is required in order to confer optimal beneficial effects. The present review focuses on the general functional, safety, and technological screening criteria for selection of ideal *Bacillus* probiotics as feed supplements as well as their mechanism of action and beneficial effects on monogastric animals for improving production performance and health status.

Keywords Probiotic · *Bacillus* · Antibiotic alternative · Feed additive · Mode of action · Gut microbiota

Introduction

Livestock production is hugely contributing to the economic growth of most countries. It has increased considerably during last decades so as to provide sufficient food for the growing world population that is expected to exceed 9 billion by 2050 [1]. The availability of enough protein sources through terrestrial and aquatic animals production is vital for meeting the growing demand for protein, an essential constituent of a balanced nutrition [2]. The extensive use of antibacterial agents at sub-therapeutical levels as growth-promoting

compounds has long been practiced in livestock production to enhance feed conversion efficacy and growth performance in commercial animal production systems [3]. However, increased antibiotic supplementation to animal feed has led to antibiotic-resistant pathogenic bacteria that menace human health because of the risk of transferring antibiotic resistance genes to the human microbiota through consumption of animal-derived products [4, 5]. From the economic point of view, emergence of antibiotic resistances has led to failure of antibiotic treatments, losses in livestock production, and increased risk of zoonotic infections [6]. In view of the increasing regulatory agencies pressure and consumer demand for safe food, the use of antibiotic growth promoters (AGPs) as feed supplements has been banned in majority of countries with Sweden being the first one to ban AGPs in 1986 [7]. Thus, to keep current production performance indicators in animal industry, several possible alternatives including probiotics, feed enzymes, prebiotic oligosaccharides, synbiotics, essential oils, and immunostimulants have

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received an overwhelming interest during the few last decades [8]. Among those substitutes, probiotics or direct-fed microbials (DFM), have generated major interest in livestock production considered as one of the fastest-growing sectors in agriculture [9]. They were defined as “non-pathogenic live microbial cells feed supplements which exert beneficial effects on both health and physiology of the target animal host, in addition to the improvement of intestinal microbiota balance, stimulation of the immune system, and overall progression in animal growth and performance” [10].

Bacillus species have a global reputation of being Generally Recognized As Safe (GRAS) microorganisms. However, some members of the species *B. cereus*, *B. alvei*, *B. brevis*, *B. circulans*, *B. coagulans*, *B. infantis*, *B. idriensis*, *B. laterosporus*, *B. licheniformis*, *B. macerans*, *B. pumilus*, *B. sphaericus*, *B. subtilis*, and *B. thuringiensis* can be pathogenic and have been linked to discomforts and diseases [11]. *Bacillus* species have the capacity to form spores withstanding harsh conditions and recently shown tremendous promise to be used as probiotics [12, 13]. Also, they are able to produce antagonistic substances against a wide range of bacterial pathogens, provide extracellular digestive enzymes (phytase, β -glucanase, xylanase, amylase, protease, β -mannanase) that enhance feed digestibility and nutrients absorption, and regulate the animal host immunity [14, 15]. Additionally, *Bacillus* DFM have proved ability to enhance both cellular and humoral immune responses through rising the number of solitary lymphoid follicles in the intestinal mucosa, controlling the development of the gut-associated lymphoid tissue (GALT), stimulating antibody responses after vaccination, and enhancing macrophage function [16]. The principal effects of probiotic additives include a remarkable resistance to pathogenic bacteria colonization and improved host mucosa immunity which can be manifested

by a reduced pathogen load, an improved health status of the animal host, and a reduced risk of contamination of animal products with harmful foodborne pathogens [17]. Also, *Bacillus* strains are forming endospores that can resist harsh intestinal conditions, survive extreme temperatures during the feed pelleting procedure, and tolerate extreme pH conditions, dehydration, high pressures, and caustics [18]. All these properties as well as their long shelf life render *Bacillus* DFM strains and their endospores an ideal feed supplement [19].

This review reports the level of general development in the impressive *Bacillus* probiotic scientific research for application in monogastrics (terrestrial and aquatic animals like poultry, pigs, and fish), with a critical assessment of principal general functional, safety, and technological screening criteria for selection of ideal *Bacillus* probiotics and their mode of action and beneficial effects on the monogastric animals. Furthermore, up-to-date *Bacillus* spore innovation technology entailing nanobiotechnology, adsorption, and surface display of proteins for improving the feed utilization efficiency is discussed.

Sources, Isolation, Identification Technique, and Probiotic Characterization of Strains Belonging to the Genus *Bacillus*

To date, many Gram-positive bacterial species including lactic acid bacteria, *Bacillus*, and *Bifidobacterium* have been reported as health-promoting probiotics in livestock production [20]. However, among these, strains selected from the genus *Bacillus* have more evident intrinsic advantages when compared to the other non-sporulating ones [21]. Indeed, they can germinate, multiply rapidly, and endure a multi-tude

Table 1 Sources of *Bacillus* probiotic strains and identification technique

Source	<i>Bacillus</i> strain	Identification technique	Animal model	References
Soil	<i>B. subtilis</i> HB2	16S ribosomal RNA gene amplification and sequence analysis	Poultry	[24]
Soil	<i>B. amyloliquefaciens</i> DSM 25,840 or <i>B. subtilis</i> DSM32324	16S ribosomal RNA gene amplification and sequence analysis	Piglets	[25]
Fermented pickles	<i>B. subtilis</i> L10 and G1	16S ribosomal RNA gene amplification and sequence analysis	Shrimp	[26]
Weaned pig	<i>B. subtilis</i> KN-42	16S ribosomal RNA gene amplification and sequence analysis	Pig	[27]
Gut of fish	<i>B. subtilis</i> ANSB060	Phenotypic characters and 16S rRNA gene sequencing	Broiler	[23]
Digestive tract of freshwater fish	<i>B. subtilis</i> KX756706 <i>B. cereus</i> KX756707 <i>B. amyloliquefaciens</i> KX775224	16S ribosomal RNA gene amplification and sequence analysis	Fish	[28]
Gastrointestinal tract of young broiler chicks	<i>B. subtilis</i> PRO	16S ribosomal RNA gene amplification and sequence analysis	Poultry	[29]

of severe environmental conditions, providing a wide range of health benefits on the host. For instance, application of *Bacillus* probiotics can enhance growth as well as immune function of animal host and help in maintaining a balanced gut microbiota [22]. Potential *Bacillus* probiotics used for monogastric animals are typically isolated from the gastrointestinal tract (GIT) of those animals or from other sources such as soil, aquatic environment, plants, and marine algae [23]. The majority of characterized *Bacillus* probiotic strains are members of the *subtilis* species or closely related ones [14]. Table 1 provides examples of sources of *Bacillus* strains used in different monogastric animal models along with their principal identification technique.

Functional Criteria for Desirable *Bacillus* Probiotic Properties

Bacillus species, which enjoy GRAS status from the US Food and Drug Administration (FDA) and are included in the Qualified Presumption of Safety (QPS) list of the

European Food Safety Authority (EFSA), have recently shown tremendous promise as probiotics [23]. Functional, safety, and technological characteristics must be considered in the selection process of probiotic *Bacillus* destined to monogastric animals as illustrated in Fig. 1. The probiotic strain must fulfill several functional aspects. For instance, it is preferred that probiotic candidate is a GRAS normal resident of the targeted monogastric gut, and capable of persisting in the GIT of that animal [30]. Also, survival to the digestive stresses, exhibition of antagonistic and anti-mutagenic properties, efficiency in immunomodulation and competitive exclusion (CE) are prerequisite [30].

To provide the expected positive effects on the monogastric animal host, probiotic *Bacillus* must have the ability to survive, colonize, and persist, at least transiently, in the host GIT [31]. Several *Bacillus* strains have been screened using in vitro as well as in vivo models for their potential probiotic functional properties [32]. *Bacillus* spores can withstand harsh environmental stresses like the extreme acidic pH of the stomach, and tolerate bile salts along with other adverse conditions within the animal GIT [32]. Besides, bacilli are

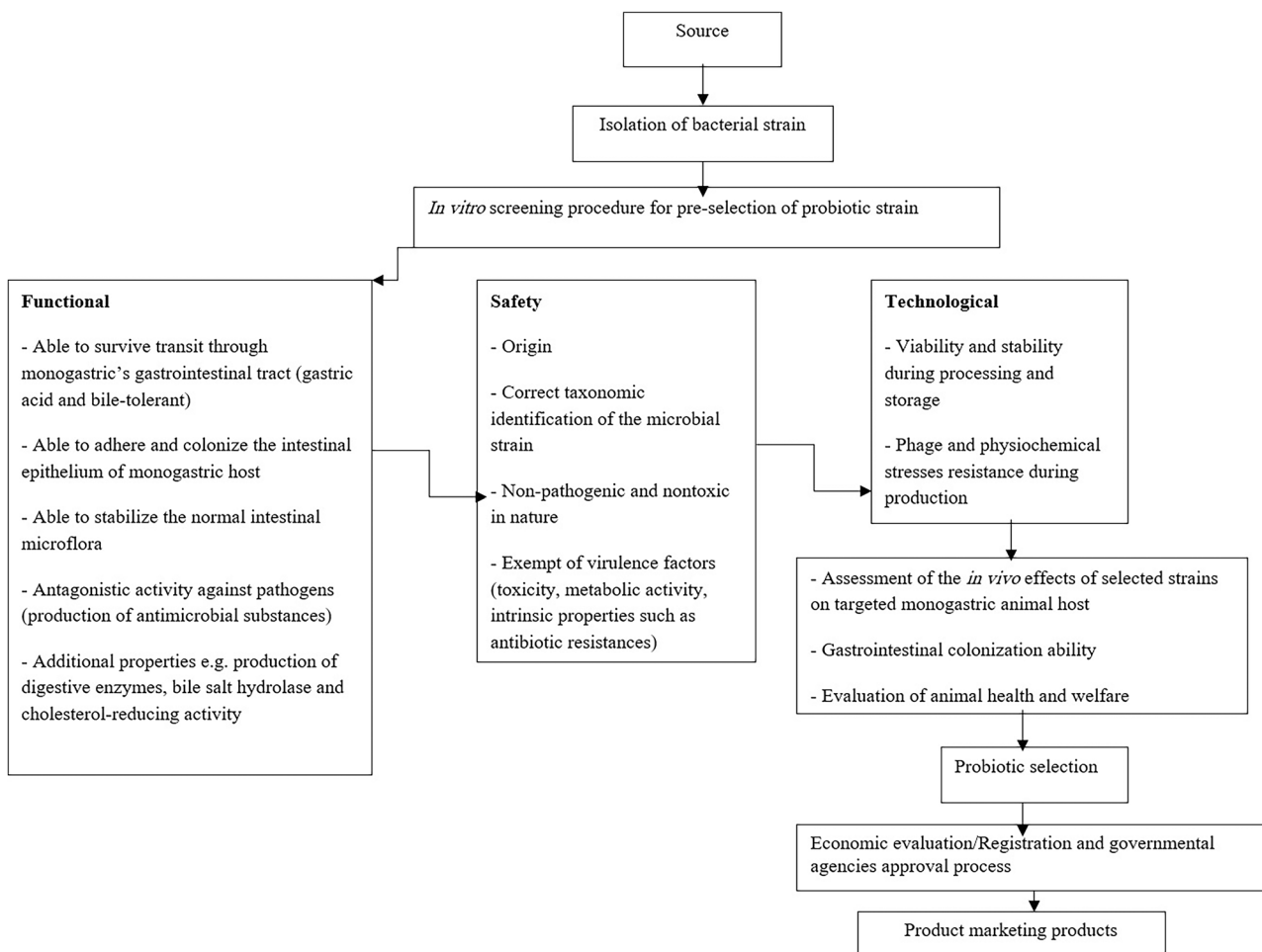


Fig. 1 Diagram providing the different criteria required for the selection of *Bacillus* strains as promising probiotics

more stable during handling and storage of food and pharmaceutical products, an advantageous property that make them pretty ingredients in formulating preparations with health promoting potential [32].

Bacillus Probiotics Induce Gut Microbiota Modulation

Ability to adhere to the intestinal mucosa is among the principal selection criteria for novel and existing probiotic strains. The property of *Bacillus* spore adhesion is essential for colonization, leading to the interaction between the probiotic and targeted host that is also linked to the ability of the strain to modulate the immune function [8]. Supplementation of *Bacillus* as DFM can positively influence host gut microbiota composition by reducing the competition for nutrients which contributes to increasing beneficial bacteria and improving GIT health [8]. Also, probiotics that belong to the genus *Bacillus* as well as the *Lactobacillus* and *Saccharomyces* genera, are among the most common supplements with proven ability to lower Firmicutes/Bacteroidetes ratio and prevent obesity [33]. Wang et al. (2017) suggested that *Bacillus amyloliquefaciens* SC06 can help to prevent obesity and its related liver damage by modifying the antioxidant capacity of swine intestinal epithelial cells and host gut microbiota [34]. In chickens, microbial colonization of the GIT starts at hatch and quickly increases [35]. Within 24 h, the microbial count in the proximal and distal intestine can attain 10^8 and 10^{10} cells/g, respectively [35]. Early supplementation with *Bacillus* spp. as a DFM is vital to maintain a durable presence within the animal's gut community [9]. Previous studies have proved that application of *Bacillus* spp. as a DFM improves in general the intestinal health and growth performance in chickens, but once more, the exact involved mechanisms are still unknown [9]. The literature reports that *Bacillus* spores are not reactive before ingestion [36]. But, they have the ability to quickly germinate once becoming within the chickens' GIT environment. The vegetative cells can develop from spores within 20 h after oral administration and could be detected all along the small intestine segments, ceca, and colon [37]. *Bacillus* DFM could influence the distribution and colonization of the intrinsic microbiota along the GIT and stimulate the growth and proliferation of other beneficial health-promoting bacteria [9]. One presented theory speculates that the growth-promoting effects of DFM in poultry production are principally linked to reduced numbering and diversity of the innate microbiota, which allows for increased nutrients absorption by the host intestinal epithelial cells and reduced detrimental effects of the microbial-derived metabolites [3].

Bacillus Probiotics Induce Gut Morphological and Immunological Changes

The probiotic-derived metabolites, DNA, and components of cell wall can contribute to the stimulation of the host immune system. This can be achieved throughout interaction with epithelial cells and effectors/regulators of the innate and adaptive immune responses like monocytes, macrophages, and dendritic cells. The immune response mechanisms might be local and restricted to the stimulation of host gut immunity such as stimulation of the production of secretory IgA (sIgA) or systemic immunity [38]. The bacterial adherence to intestinal enterocytes is not only helpful for gut colonization, but can as well boost the gut-specific lymphoid tissue immune cells [39]. Previous reports strongly suggest that spores of *B. subtilis* are able to translocate through microfold cells that are located in the epithelium covering mucosa-associated lymphoid tissues like the Peyer's patches (PPs) of the small intestine prior to be transported to efferent lymph nodes [40]. These PPs are rich in antigen-presenting cells including dendritic cells and macrophages, that are widely contributing to antigens processing and presentation to B cells allowing the production of sIgA [41]. Stimulation of sIgA production as the principal antibody class in mucosal secretions is crucial for neutralization of pathogenic microorganisms [41].

On the other hand, various studies have revealed that spores of *Bacillus* can similarly stimulate phagocytosis mediated by macrophages that are a cornerstone of the innate immune system [42]. It is well documented that the immune function modulation or the host immune systems stimulation are vital characteristics of possible probiotic candidates and are linked to their antagonism potential [32]. These antagonistic effects of probiotics are indispensable to stop or lower pathogenic infection [32]. *Bacillus* DFM can interact directly with the host to generate a beneficial immune response against harmful pathogens at the intestinal epithelium of chickens [9]. Good gut health plays a key role in enhancing effectiveness of poultry production as it directly correlates with improved growth and production performances [9]. Many factors linked to disease and stress are able to cause disruption of intestinal epithelial integrity that consequently reduces nutrients absorption efficiency and increases the risk of pathogenic invasion and inflammatory diseases development. Thus, a notable reduction in growth performance is expected [43]. Animal's gut barrier function should be preserved in order to protect the host from dangerous biological or chemical invading agents [44]. This can be achieved by two major mechanisms including the epithelial goblet cells-mediated secretion of the protective mucus blanket [45]. The mucus is composed mainly of

mucin glycoproteins responsible for the viscous properties, water, salts, lipids, and several proteins exhibiting protective functions [46]. DFM, such as those belonging to the genus *Bacillus*, can induce the expression of *MUC2* gene responsible for mucin production, to neutralize the inflammation triggered by pathogenic germs [46]. When inducing an inflammatory response in chickens by intraperitoneal injection of lipopolysaccharide (LPS) from *Escherichia coli* [47], the group which was fed with the probiotic *B. subtilis* 1781 strain showed significant increase in the ileum *MUC2* expression levels [48]. This up-regulation corroborated with remarkable reduce in inflammation signs as observed by decreased α -1-acid glycoprotein (α -1-AGP) expression, that is frequently associated with severe colitis [48]. In addition, the up-regulation of *MUC2* expression was found to be also probably induced in response to the LPS circulating in chicken blood, and not exclusively just as a result of the direct contact with intestinal epithelium [48].

Metabolic Features of the Genus *Bacillus*

The antagonistic activities are of major importance in the procedure of potential probiotics screening [49]. These activities are tightly associated with the ability to release antimicrobial agents supporting CE of pathogens [20]. The term “CE” is defined as the aptitude of desirable probiotic bacteria to enter in competition with undesirable gastrointestinal pathogens for adhesion to intestinal surfaces [50]. For instance, among the desirable probiotic properties of *B. subtilis* KMP-N004 is the inhibition of pathogens adhesion to porcine enterocytes [51]. *Bacillus* strains are known as potent producers of a wide variety of antimicrobial substances among them, lipopeptides like surfactin, iturin, and fengycin; bacteriocins; and bacteriocin-like inhibitory substances [52, 53]. In general, these antimicrobial compounds are principally efficient against Gram-positive bacterial pathogens, but few of them also show antagonistic potential toward some pathogenic Gram-negative bacteria and fungi [54].

On another hand, it is well documented that the recent tremendous potential of *Bacillus* species as probiotics is tightly linked to their inherent capacity to produce spores from initial vegetative cells [55]. They possess several advantages over other non-spore formers such as members of the genus *Lactobacillus* [55]. Indeed, *Bacillus* spores are naturally stable and able to resist harsh environmental stressful conditions and transitions during storage and handling without any loss in their vital potential [56]. This includes high temperatures of the feed pelletization procedure (around 80 °C), extreme pH, dehydration, and high pressures [57]. In addition, vitality of *Bacillus* spores is often not compromised by bile and acidic pH values encountered in the GIT

of monogastrics compared to vegetative cells [58]. Several reports have claimed that ingested *Bacillus* spores are able to germinate in the intestinal compartment and may exert their expected beneficial effects on the monogastric animal host through the production of wide range of bioactive metabolites, including the release of antimicrobial compounds supporting CE of pathogenic bacteria [23]. The adhesion to intestinal epithelial cells of the host is among the key advantageous properties examined during the procedure of probiotic candidates screening [59]. Generally, the spores of *Bacillus* are characterized by a higher hydrophobicity and superior adhesion ability when compared to the vegetative cells living form [60]. A correlation between surface hydrophobicity and good adhesion capacity of *Bacillus* isolates has also been reported [61]. The superior hydrophobicity of spores is most likely attributable to the existence of surface-associated proteins that probably play key role in the adhesion property of probiotic strains to the GIT [23].

In addition to spores formation ability and production of antimicrobial compounds, several *Bacillus* probiotic strains are able to liberate directly in the lumen of the host digestive tract, a variety of extracellular digestive enzymes including phytase, xylanase, β -glucanase, β -mannanase, amylase, protease, and lipase [56]. These enzymes can improve feed conversion ratio (FCR) by reduction of anti-nutritional factors in plant-derived feedstuffs like non-starch polysaccharides (NSP), enhancement of feed ingredients digestibility, and absorption of nutrients [62]. While the principal interest of using these enzymes is the improvement of digestion, some of them can also be implicated in the lysing of the cell wall components of pathogenic microbes which in turn causes the global resistance of the animal host against these pathogens [63]. It is well known that the abundance of NSP in diets increases the host intestinal viscosity and affects negatively the digestibility, the nutrients absorption, and growth performance [64]. Neutralizing the adverse effects generated by NSP can be achieved by supplementation of animal diet with the corresponding microbial enzymes or a multi-enzymes producing DFM [65].

Safety Criteria for Selection of Promising *Bacillus* Probiotic Strains

Safety aspects that probiotic candidates must meet include specifications like origin (healthy relevant animals’ GIT) as well as lack of any traits of pathogenicity, nondigestive discomforts, and heritable antibiotic resistance genes [58]. The identity of microorganisms is usually ascertained primarily before evaluating them for their probiotic attributes [66]. Microbes are subjected to a battery of morphological, biochemical, and molecular tests to assign them to the corresponding genus and species [67]. In general, the 16S rDNA

gene sequencing proved to be insufficient to easily distinguish between *Bacillus* species that are closely related [68]. Analysis of additional molecular markers like *gyrA* or *gyrB* genes can be helpful [68]. Closely related bacterial isolates from the same species are further differentiated to sub species and strain level using newer and established techniques, such as DNA fingerprinting, sequencing of selected genes, oligonucleotide probes, pulse-field gel electrophoresis etc. [69]. Other molecular techniques, including denaturing/temperature gradient gel electrophoresis and fluorescence in situ hybridization, are utilized to identify and characterize probiotic strains [70]. Recent development in whole genome sequencing technologies allows precise taxonomic identification of potential *Bacillus* probiotic candidates and especially provides information about the presence of antibiotic resistance genes and virulence factors as well as the possible pathogenicity toward monogastric host [71].

To confirm that probiotic products are safe to consume and safeguarding health, regulatory quality control, and strict rules are required [71]. Among these, the disadvantage of toxin production that can be detected in some *Bacillus* strains is a main safety problem for their administration to animal hosts [32]. *B. cereus* is one of the more important causes of food poisoning. It produces one emetic toxin and different enterotoxins [72]. The diarrheal syndrome is caused by the production of three different heat-labile enterotoxins all through vegetative growth of *B. cereus* in the small intestine [72], whereas the emetic-type illness is caused by a heat-stable emetic toxin called cereulide, produced under various conditions during the growth of *B. cereus* in the food itself [73]. *B. cereus* also has the ability to release various other kinds of toxins such as enterotoxin T, enterotoxin FM, and cytotoxin K [72]. In addition to the well-documented previously cited toxins, many other virulence factors including hemolysins and cerolysins are also involved in the toxic activity of enterotoxins from *B. cereus* strains [74]. Besides the well-known pathogenic *B. cereus*, sporadically other species from the *Bacillus* genus showed the ability to release metabolites exhibiting significant toxicity towards mammalian cells, such as the heat-stable toxin amyloisin, that can be liberated by bacilli associated with food poisoning like *B. subtilis*, *B. amyloliquefaciens*, and *B. mojavensis* [75]. During the screening strategy, strains showing cytotoxicity are not endorsed for use as probiotic candidates [76].

Another threat that should be seriously considered is the capacity of some *Bacillus* isolates to hold transmissible antibiotic resistance determinants, especially resistance genes that are carried by plasmids as well as mobile genetic elements such as transposons and insertion sequences [77]. Horizontal gene transfer within the host GIT might occur between probiotic and opportunistic or pathogenic

bacterial strains. For instance, aminoglycosides, macrolides, β -lactams, and chloramphenicol resistance genes have been detected in *B. clausii* DSM8716 probiotic strain [78, 79]. The tetracycline resistance gene *tet M* that belongs to the *B. cereus* group, was found to be carried by Tn916-like transposon that could be transmitted to *B. subtilis*, *Staphylococcus aureus*, and enterococci [23]. Besides, the *tet K* gene conferring resistance to chlortetracycline in *B. subtilis* was carried by a plasmid which could be received by *E. coli* via conjugative transfer [80]. Transfer of genes conferring resistance to antibiotics between bacteria contributes to the development of acquired antibiotic resistances which result in the failure of treating certain infections and economic losses in animal production systems [81]. For this reason, the EFSA has recommended that the *Bacillus*-based products intended for use as feed supplements should be checked for sensitivity to antibiotics commonly used in human and veterinary medicine to avoid any resistance spreading [82]. The antimicrobial resistances that should be checked in *Bacillus* species include for example, vancomycin, tetracycline, chloramphenicol, gentamicin, kanamycin, streptomycin, erythromycin, and clindamycin [82].

Technological Requirements to Assess Probiotic Strain Viability and Stability

In addition to the functional and safety aspects, probiotic bacteria must also meet the technological criteria linked to the production and processing of animal feed. Moreover, resistance to in vitro as well as in vivo conditions after administration of the probiotic is important [83]. For example, the probiotic microorganism should not be killed by the host's defense mechanisms and able to withstand the specific conditions occurring during the transit in the monogastric host [83]. A major quality criterion that must be met in the manufacture, regulation, and marketing of DFM is the precise definition of the number of viable cells in the feed additive product in accordance with the label [84]. A variety of methods can be used to determine the bacterial concentration in commercial preparations. These include traditional plating assays, optical density measurements (turbidity), direct counting methods like flow cytometry, quantitative polymerase chain reaction, cell mass determination, and measurements of cellular activity [85]. Depending on the precise product matrix, one assay technique can be chosen for better precision and reproducibility [86]. On the other hand, economical evaluation of probiotic preparation and endorsement by an official certification service must be done prior to large scale production and commercialization. A

diagram that summarizes the diverse criteria that *Bacillus* probiotic strains must meet in order to be considered beneficial feed supplements is shown in Fig. 1.

Modes of Action and Beneficial Effects of *Bacillus* for Monogastric Animals

Probiotic bacteria from the genus *Bacillus* are expected to balance the gut microbiota generating beneficial activities to the animal host. We classified the modes of action of these DFM in the following:

Competition for Adhesion to Epithelial Layer

The mechanism by which probiotic bacteria compete for intestinal adhesion sites is designated as “CE.” Among the main properties required for selection of adequate *Bacillus* DFM candidates are the strain ability to adhere to the epithelial surface, colonize the monogastric host gut, and subsequently form a barrier on the intestinal surface to prevent the adhesion of pathogenic microorganisms [87]. A recent study has demonstrated that *B. subtilis* C-3102 supplementation over a period of 3 weeks accelerates exclusion of the pathogen *Salmonella enterica* from the cecum, liver, and spleen of the chickens in a dose-dependent manner [88]. Many *Bacillus* probiotic candidates have shown excellent in vitro adhesion efficacy. For instance, the *B. amyloliquefaciens* US573 was found to exhibit good adhesion efficacy to chicken enterocytes collected from the duodenum, jejunum, and ileum [89]. In addition, Gu et al. (2015) have observed that the *B. coagulans* CGMCC 9951 strain exhibited stronger adhesion to pig intestinal mucus than that of *B. subtilis* JT143 and *L. acidophilus* LY24 strains isolated from two commercial probiotic preparations [90]. High in vitro adhesion to mucus was also shown for the *Bacillus amyloliquefaciens* COFCAU_P1 strain isolated from the digestive tract of a freshwater teleost rohu (*Labeo rohita*) [91]. Mucus-binding and surface-associated proteins are among the principal determinants involved in the adhesion as revealed by genome sequencing of many *Bacillus* probiotic strains [90].

Production of Inhibitory Substances

Many *Bacillus* species are renowned producers of antimicrobial peptides, especially bacteriocins which are small ribosomally synthesized peptides released for bacterial antagonism in a given natural ecosystem [92]. These *Bacillus*-derived bacteriocins affect principally Gram-positive

bacteria by binding onto their outer membranes for disruption of the membrane biosynthesis [93]. Bacteriocins can serve as colonizing peptides by facilitating the installation of probiotic strain into an already occupied niche on the intestinal epithelium. The competitive advantage confer to the probiotic *Bacillus* the capacity to invade host intestine surfaces [94]. Generally, bacteriocins produced by strains that are members of the genus *Bacillus* have the reputation to exhibit a wide antibacterial spectrum and to be active under extreme temperature and pH conditions [95]. For instance, *B. amyloliquefaciens* ZJHD3-06 strain isolated from the marine fish *Epinephelus areolatus* produces a novel bacteriocin namely “CAMT2” that has antagonistic activity against *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Vibrio parahaemolyticus* [96].

Immune System Modulation

Modulation of immune system is one of the most common benefits linked to dietary probiotic inclusion. The observed positive effects are depending on the monogastric animal, host immune system, probiotic strains used, and conditions of application [97]. For example, improvement in immune function reflected by serum IgA and IgM increases was noted in chickens fed with diets containing a mixture of *B. subtilis* BYS2 and BG5 [98]. Similarly, administration of *B. subtilis* promoted the increase in IgA concentrations in pigs [99]. Reduce or prevention of intestinal inflammatory reactions has been also observed in poultry following the administration of *B. subtilis*-based probiotics [100]. Regarding fish species, increase in lysozyme activity is among the noted beneficial effects of probiotic supplementation on immune system modulation. Lysozyme is an important defense protein (non-specific immunity). It has the ability to lyse the cell walls of Gram-positive bacteria and plays a key role in combating infection in fish. Many studies have shown that inclusion of different *Bacillus subtilis* strains in diets of various fish species increases mucus lysozyme activity. For instance, hybrid grouper fed with *B. subtilis* 6–3–1 strain exhibited significantly higher lysozyme activity level than fish fed with a control diet without probiotic supplementation [101].

Production of Extracellular Hydrolytic Enzymes

Currently, members of the spore-forming *Bacillus* genus are considered among the main sources of DFM destined for monogastric animals [102]. They have the ability to produce a wide range of extracellular enzymes that actively contribute to enhancement of digestion (carbohydrates hydrolases and proteolytic enzymes) and nutrients absorption [103]. A previous study reported that the selection of *Bacillus* DFM

was depending on in vitro enzyme secretion profiles, which associated with reduce in supernatant viscosity and proliferation of *Clostridium perfringens* [62]. It was suggested that the extracellular enzymes which are secreted by the DFM could reduce the quantity of available nutrients that are required for growth of *C. perfringens*, stimulating the rate of transit of the digesta through the GIT and improving the intestinal microbial environment [104]. Cereal grains are among the regularly used ingredients in livestock animal feed. They are rich in anti-nutritional factors including NSP, resistant starch, and non-digestible proteins that are weakly digested by monogastrics. Additionally, the NSP can also exert anti-nutritive effects by chelating vital minerals like calcium, iron, and magnesium, reducing nutrients absorption as a result of increased ileal viscosity, and altering the host gut microbiota. Digestive enzymes such as xylanases, amylases, and proteases are regularly supplemented to animal feed to improve growth performance by enhancement of digestibility and alleviation of the anti-nutritive effects of poorly digested feed ingredients [105]. Additionally, the hydrolysis products of animal feedstuffs generated by exogenous enzymes used as feed supplements can contain substances like oligosaccharides that may promote the growth or metabolic activities of beneficial bacteria. Altogether, these observations suggest that the administration of particular enzyme blends can confer an additional advantage when combined with suitable DFM. Table 2 shows the main involved enzymes and their functions.

Application of Probiotics in Livestock

In Poultry

Bacillus probiotics are commonly utilized in the poultry sector. They can improve the growth and production efficiency

of poultry birds, protect against pathogenic microbes, enhance the immune system responses, strengthen bones, and assist in fighting off parasite infections. Infection of chickens with some pathogenic bacteria including *Salmonella* spp., *Clostridium perfringens* and *Campylobacter jejuni* increases the risk of contamination at the different stages along the food chain resulting in a dangerous condition simultaneously for poultry and human. Therefore, probiotics can act as a natural alternative against of *Campylobacter*, *Salmonella*, and *Escherichia coli* in the preharvest stages [58]. For instance, the number of Enterobacteriaceae in the excreta decreased following *B. subtilis* C-3102 strain administration [112]. Feeding of broilers with this *B. subtilis* strain also causes a decrease in shedding of *Campylobacter* [113]. As well, dietary supplementation with either *B. subtilis* DSM17299 or *B. cereus* var. leads to significantly reduced *Salmonella* colonization [23]. Furthermore, *B. subtilis* PB6 addition to broilers causes a decreased number of *C. perfringens* in the intestine [114]. In addition to preventing gut colonization by the pathogen, feeding the poultry with *Bacillus* probiotic can enhance the FCR and improve weight gain. Other benefits for *Bacillus* as probiotic include the effects on broilers intestine histology and morphology, as the increase in the villus height as well as the villus height to crypt dept ratio. These effects, contribute to improving digestibility and increasing intestinal nutrients absorption capacity [115]. Broilers fed diets supplemented with *Bacillus* enzyme-producing probiotics showed decreased digesta viscosity that is caused by soluble NSP, which are known by their negative impact on availability and absorption of nutrients [116]. Besides enhancing the growth performances and promoting health of poultry, feed supplementation with *Bacillus* could also improve the meat quality (increase in meat lightness, redness and yellowness) [117] and eggs (increase in eggshell strength and thickness, yolk color score, Haugh unit, and decrease in cholesterol content of yolk) [118]. *Bacillus* can limit influence from toxin

Table 2 Main enzymes secreted by *Bacillus* strains and their functions in monogastric animals

Enzyme	Producer strain	Substrate	Function	Reference
Protease	<i>Bacillus cereus</i> AT	Proteins	Contributes to proteins digestion and improves FCR and weight gain	[106]
Lipase	<i>Bacillus methylotrophicus</i> PS3	Lipids	Digests lipids	[107]
Cellulase	<i>Bacillus subtilis</i> BY-3	Cellulose	Hydrolyzes cellulose, and enhances fiber degradation	[108]
Xylanase	<i>Bacillus subtilis</i> E44	Xylans	Contributes to degradation of NSP as well as release of nutrients and decreases intestinal chyme viscosity increasing animal production efficiency	[106]
Phytase	<i>Bacillus subtilis</i> US417	Phytic acid	Releases phosphorus (P) from phytates contributing to better bioavailability of P and vital minerals and enhancement of growth performance and health of animals along with environment protection	[109]
Keratinase	<i>Bacillus subtilis</i> S14	Keratin	Degrades keratin and improve digestion	[110]
Mannanase	<i>Bacillus amyloliquefaciens</i> 10A1	Mannans	Contributes to degradation of NSP and decreases intestinal chyme viscosity increasing animal production performance	[111]

in contaminated broilers feed and decrease emission of NH_3 from poultry manure [23]. The increase in gut permeability caused by several enteric pathogens such as *Salmonella* spp results damage in the intestinal tight junction that referred as “leaky gut” [119]. With inflammation, the leakage of serum fluorescein isothiocyanate dextran (FITC-d) increases which is judged as indicator to estimate enteric inflammation produced with gut permeability in broiler chickens [120]. During therapeutic study, the observed significant decrease ($p < 0.05$) of serum FITC-d level by DFM administration as compared to the untreated control group resulted in the reduction of negative effects of *S. Enteritidis* by increasing the tight junction proteins organization [121]. The vital role of antioxidant enzymes like super oxide dismutase (SOD) includes the degradation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anions that are produced during the inflammatory process [122]. A significant ($p < 0.05$) increase of SOD activity was observed in the control group of the prophylactic and therapeutic study, when compared to the group fed with the DFM [121]. The increase in SOD activity in the control group could be linked to the reaction to increased oxidative stress attributable to severe intestinal damage caused by *S. Enteritidis* [121]. Also, the large increase in the amount of IgA ($p < 0.05$) observed in both in vivo studies may be related with disruption of the intestinal epithelium, as intestinal IgA secretion acts as the first line of defense in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms, counteracting inflammatory processes and boosting the mechanism of non-specific defense [123]. On the other hand, as a consequence of DFM administration, the decrease in SOD activity as well as IgA level may be attributed to its anti-inflammatory and immune modulating properties to reduce the harmful effects of *S. Enteritidis*. This can minimize the intestinal morphological and immunological changes by cytoprotective proteins and modulation/regulation of cytokines expression. In addition, with implied health benefits for birds, the DFM was able to sustain bacterial species in the intestine while reducing potentially harmful populations. Hernandez-Patlan et al. (2019) showed that the addition of *B. subtilis* DSM 32,315 in broilers feed may stabilize the microbiome and prevent opportunistic pathogens like *Campylobacter jejuni*, *E. coli*, and various *Salmonella* spp. from other intestinal disorders [104]. The possible impacts of feeding with *B. subtilis* DSM32315 strain on physiological improvements and GIT function and immune system should be investigated to explain other potential actions of the probiotic that may have involvement in promoting health and increasing growth performance of poultry [104].

In Pigs

Similar to poultry, newborn pigs possess a sterile gut and gradually develop their typical microbiota through contact with their mother and the surrounding environment. The cycles after birth and post-weaning are the most susceptible times in the pig’s life in which the removal of in-feed antimicrobial agents could have an effect on mortality [124]. Pigs are particularly sensitive to gut invasion by bacterial pathogens responsible for growth decrease and diarrhea during those cycles [125]. Furthermore, weaning exposes the piglets to a biological stress that can lead to dysfunctions of the intestinal and immune systems and can lead to decreased feed intake and growth along with altered pig health, particularly in the first week after weaning [126]. It has been claimed that during the first week after weaning, the microbiota in the pig gut fluctuates, and a duration of 2–3 weeks post-weaning is needed to stabilize gut microbes and completely improve their fermentation ability [127]. During these life stages, the piglet must be exposed to a shielding gut microbiota that would defend towards environmentally acquired pathogenic germs by both direct and indirect means (stimulation of host immune system), with the additional benefit of increasing the digestibility and absorption of nutrients [128]. Probiotic administration has been reported to influence in a positive way the feed intake and average live weight with greater neonatal piglet size and vitality at the same time [58]. Supplementation of pigs feed with probiotics can result in an improvement in meat quality attributes like color, marbling, tenderness, juiciness, and flavor [58]. In addition, pathogenic bacteria (*E. coli*, *Salmonella*, *Clostridium difficile*, *Clostridium perfringens*, *Listeria*), parasites (*Isospora*, *Cryptosporidium*), or viruses (*Coronavirus*, *Rotavirus*), which are triggering growth reduction and diarrhea, are more susceptible to intestinal colonization from birth to post-weaning piglets [127]. Many studies have assessed the effectiveness of probiotics during this episode and recommended the use of probiotics in this period [58]. *Bacillus* probiotic strains have been widely used in pigs. They can help to improve host gut health and immunity and reduce environmental pollutants like odor gas emissions from pig manure. For instance, inclusion of *Bacillus subtilis* H4 strain as feed additive resulted in a decrease in scouring in weaned pigs challenged with enterotoxigenic *E. coli* K88. Also, supplementation of basal diets for growing-finishing pigs with BioPlus 2B (*B. licheniformis* and *B. subtilis*) resulted in a decrease in morbidity and mortality, increase in fattening pig performance parameters and improve of carcass con-

sistency [23]. In addition, this combination of *B. subtilis* and *B. licheniformis* spores decreased body weight of gestating sows, reduced prevalence of diarrhea and mortality in piglets, increased weaning litter weight and growth rate of young piglets [58]. Moreover, the use of *B. cereus* as probiotic conferred not just a lower incidence of diarrhea in pigs, but also a better FCR [58].

In Fish

In aquaculture, dietary supplementation of probiotics offers an environmentally friendly preventive prophylactic measure in terms of fish growth performance and health [129]. Probiotic strains that belong to the genus *Bacillus* are thought to be important tools for promoting sustainable aquaculture owing to their significant beneficial effects for aquaculture industry. They exert positive effects through multiple mechanisms like promotion of growth performance by production of enzymes increasing efficiency of feed utilization, suppression of disease invasion by stimulation of immunity, and improvement of aquatic ecosystems for growth and reproduction of fishes [8]. For instance, optimum supplementation of probiotic *Bacillus* species to *Oreochromis mossambicus* and *Lates calcarifer* can increase the specific growth rate and decrease the FCR. *Bacillus* can contribute to increasing the fecundity, survival rate, number of red and white blood cells in blood, stress, and disease resistance. Also, administration of *Bacillus* probiotics can stimulate the activity of intestinal enzymes contributing to efficient digestion in fish [129]. On the other hand, by secretion of enzymes or release of auto-inducer antagonists, certain probiotic bacteria including *B. cereus* strains permit degradation of pathogen signaling molecules (quorum quenching) [130].

In aquaculture, the administration of *Bacillus* probiotic strains can enhance the disease resistance against several bacterial pathogens. Obviously, following diet supplementation with *Bacillus*, an increased resistance against *Streptococcus iniae* [131], *Aeromonas hydrophila* [132], *Acinetobacter* sp. and *Acinetobacter tandoii* [28], and *Aeromonas salmonicida*, *Streptococcus agalactiae*, *Lactococcus garvieae*, and *Vibrio parahaemolyticus* [133] has been recorded. The improvement in health status and disease resistance by dietary administration of *B. subtilis* probiotic strains has also been observed in different aquatic species including rainbow trout [134] and tilapia [135]. Administration of *Bacillus* strain TC22 (at 10^9 CFU g^{-1} of feed) alone or in combination with prebiotic fructo-oligosaccharide at 0.5%, resulted in increased phagocytosis, respiratory burst, and phenoloxidase activity of sea cucumber *Apostichopus japonicas* and disease resistance against *Vibrio splendidus* infection [136]. *Bacillus* sp. (PM4) and *B. subtilis* from a commercial product that demonstrated high inhibitory activity towards *V. mimicus* and *V. cholera* non-01, while

B. mycoides, isolated from marron inhibited just *V. mimicus* [137]. Recently, *B. subtilis* WS1A strain originating from a Bay of Bengal sponge demonstrated antagonistic activity on fish bacterial pathogens [138].

Probiotic bacilli have been reported to greatly improve the quality of water by preserving the equilibrium of several physicochemical parameters, regulating of the composition and abundance of microbial species by antagonism, decomposing various organic waste, and keeping the ecosystem safe for aquaculture [139]. It is evident that *Bacillus* has a huge potential to contribute to the sustainability of fish farming by improving water quality and preserving the total welfare of cultivated fish such as growth improvement, feed usage, immune system responses, and defense against infectious diseases, especially infection by pathogenic bacteria [8]. The current state of *Bacillus* probiotics use in aquaculture needs intensive further research and vigilant application.

***Bacillus* Spore Innovation Entailing Nanobiotechnology, Adsorption, and Surface Display of Proteins for Improving Feed Utilization Efficiency**

Bacillus subtilis has the ability to adapt to severe nutrients scarcity by entering in a complex differentiation process, resulting in the development of highly resistant spores. The spore consists of a central core compartment containing a clone of the chromosome and is enveloped by a dense layer known as the cortex, a transformed type of peptidoglycan. A multilayered protein coat covered the cortex. It is formed by an inner layer attached to the cortex in addition to an outer layer of a protein structure that protects the spore from toxic chemicals, lytic enzymes, and protozoan phagocytic predation [140]. The characteristics of coat layers of *Bacillus* spores have recently qualified them as emerging nanostructure that provides a fresh and fascinating surface to display several biomolecules [141]. Since *B. subtilis* has a clear safety record (GRAS) as an additive in human as well as animal preparations, display of functional molecules like enzymes on spore coat has great potential of application in the field of probiotics [142]. The display of feed enzymes on the *Bacillus* spore surface can guarantee effective implementation of enzymatic action in situ at a reasonable expense. Xylanase, hemicellulase, cellulase, protease, glycanase, or phytase are examples of feed enzymes that are candidates for display [140]. *Bacillus* strains are not only important as probiotics for animal farming, but may also be of use as a vehicle system for a number of molecules in so-called nanobiotechnology [23]. Recently, the extensive developments in molecular biology as well as cell biology have contributed to the identification of a wide range of large bacterial and viral antigens [143]. However, the translation of such antigens into efficient vaccines is con-

ditioned by a delivery mechanism that is able to avoid loss of biological activity that sometimes impairs the potency of antigen [143]. Recently, *Bacillus* spp. spores have proven their ability to be used to design vaccines for the next generation [144]. The bacterial spore is a cell form that provides a potentially useful method for delivering antigens to mucosal surfaces [143]. Spore has unusual properties of resilience and can withstand high temperatures, dehydration, and exposure to solvents and additional toxic chemicals [143]. These special features make the *Bacillus* spore an attractive vehicle for the delivery of heterologous proteins, enzymes, antigens, and other bioactive molecules to targeted niches, such as the GIT [145]. Besides, the spores of recombinant *B. subtilis* have been described as a possible bioremediation device for adsorption of ions like nickel [146]. The CotB spore surface protein was developed to express eighteen residues of histidine inside the spore coat [146]. When tested to determine their efficacy in adsorbing nickel ions, recombinant spores proved to be slightly more successful in metal-binding than wild type ones [146]. The simple purification process, high robustness, and remarkable efficacy of *B. subtilis* spores in nickel binding render the recombinant spore an innovative and effective instrument with promising potential for the bioremediation of polluted environments [146].

Conclusion

The aim of the present study was to determine the beneficial effects of *Bacillus* probiotics administration in monogastric animals. The use of *Bacillus* DFM in these animals is increasingly growing, as seen by the increase in research studies investigating the regulation of gut microbiome and immune activation in these hosts. The ability of *Bacillus* species to survive feed processing and provide the host gut with their benefits is an advantage over several other widely studied non-*Bacillus*-based DFM, in addition to their commercial availability for large-scale use. There is an evidence that through decreasing the excessive growth of bacteria in the small intestine via the development of antimicrobial peptides, *Bacillus* DFM decrease competition for nutrients, encourage the proliferation of beneficial bacteria and production of bioactive metabolites, and change the immune system response to the host's advantage. All these are examples of modes of action that culminate in promoting the animal growth. Although the beneficial effects of various *Bacillus* bacteria are not identical, the next mechanisms of action elucidation steps may comprise the identification of global metabolites developed to understand the relationship between supplemented probiotic and host bacteria in the presence of well performing *Bacillus* DFM.

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Declarations

Conflict of Interest The authors declare no competing interests.

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