Chapter

B. subtilis Probiotics for Humans, Animals and Plants: Mechanisms, Applications and Prospects

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Abstract

Bacillus bacteria have increasingly attracted research interest due to their promising probiotic properties, including strong antimicrobial, antidiarrheal, and immunostimulatory effects. They are able to stimulate the growth of natural flora, prevent intestinal inflammation, and demonstrate excellent stability even under adverse conditions. This chapter explores the probiotic applications of *Bacillus subtilis* strains in humans, animals, and plants. First, the chapter reviews the probiotic mechanisms of *B. subtilis* and the current state of research in this area. The potential applications of recombinant *B. subtilis* probiotics are also covered, along with results from preclinical studies and the future role of *B. subtilis* probiotic therapy in clinical settings. This chapter aims to survey the current landscape of *B. subtilis* probiotic research and hypothesize about its potential future impact.

Keywords: *B. subtilis*, probiotics, recombinant probiotics, biotherapy, live therapeutics

1. Introduction

The worldwide use of probiotics has increased due to their positive effects on the human microbiota and health [1]. Probiotics, as defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) panel in 2013, are live microorganisms that provide health benefits to the host when administered in sufficient quantities [2]. Most human dairy products contain probiotic bacteria, and the global market for probiotic supplements is growing significantly. *Lactobacillus* and *Bifidobacterium* are the most commonly used and commercialized genera [3, 4]. However, the genus *Lactobacillus* is microaerophilic, whereas the genus *Bifidobacterium* is strictly anaerobic. Furthermore, these genera are slow-growing and sensitive to temperature and gastric juices, leading to challenges in production, storage, and delivery processes [5]. Among these genera, the species *Bacillus subtilis* probiotics provide multifaceted advantages over conventional probiotics in several areas due to their advanced properties, including shelf stability, resistance to extreme environmental conditions, and safe interactions with humans, animals, and plants [6]. Moreover, *B. subtilis* has several distinctive characteristics, such as versatility in nutrient use for growth, spore formation, a high growth rate, growth in both aerobic

and anaerobic environments, and high enzyme and antimicrobial production, making it an attractive bacterium for probiotic research [7].

Bacillus species have been utilized as probiotics for at least 60 years, with the 1958 registration of the Italian product Enterogermina as a medicinal supplement [8]. Of the *Bacillus* species, endospore-forming soil-dwelling *B. subtilis* is the most ubiquitous bacterium in diverse ecological settings ranging from terrestrial to aquatic environments [9]. This organism's genetics and physiology have been extensively researched, and the understanding of its genetic background and physiology is second only to that of *Escherichia coli* [10, 11]. Recent probiotic studies of *B. subtilis* have made significant advances in clarifying the spectrum of activity that makes this bacterium one of the most appealing probiotics [5]. *B. subtilis* strains have been studied as probiotics for their antibacterial, antifungal, antiviral, antidiarrheal, and immunostimulatory effects, as well as for their potential to compete with pathogens for survival, inhibit intestinal inflammation, and stimulate the growth of normal intestinal flora [12–17]. This chapter discusses the applications of *B. subtilis* probiotics in humans, animals, and plants. First, the chapter reviews the probiotic mechanisms of *B. subtilis* and the current state of research in this area. The potential applications of recombinant *B. subtilis* probiotics are also be covered, along with results from preclinical studies and the future role of *B. subtilis* probiotic therapy in clinical settings.

2. Mechanisms of action of *B. subtilis* probiotics

B. subtilis' probiotic effects on the host can be attributed to a variety of mechanisms, including antimicrobial effect, antidiarrheal effect, immunostimulatory effect, competitive exclusion of pathogens, prevention of intestinal inflammation, and stimulation of growth of intestinal normal flora [18].

2.1 Synthesis of antimicrobial agents

Both industry and healthcare have made extensive use of *Bacillus* spp. due to their ability to produce a wide range of biologically active metabolites, particularly antimicrobials [19]. Pathogenic infections are a leading cause of morbidity and mortality worldwide, and while synthetic antibiotics are used to control pathogenic overgrowth, their indiscriminate use leads to the emergence of multidrug-resistant pathogenic bacteria. *B. subtilis*, one of the most prominent species, has shown promise in this area by serving as a viable therapeutic antimicrobial, as it synthesizes 66 antibiotics and allocates 4–5% of its genome to antibiotic biosynthesis [1, 7, 20]. The antibiotics synthesized by *B. subtilis* have diverse structures and antimicrobial properties. Specifically, *B. subtilis* synthesizes antimicrobial agents that disrupt cell walls and plasma membranes and interfere with intracellular processes [19]. Bacilysin exerts its effect by targeting the cell walls of *Candida albicans* and foodborne pathogens such as *Escherichia coli*, *Salmonella enterica* and *Bacillus cereus* [21, 22]. *B. subtilis* biosynthesizes lipopeptide antibiotics such as surfactin and fengicyn, which exert their antimicrobial activity against harmful microorganisms such as plant pathogens such as *Rhizopus stolonifer* through their interaction mechanisms with phospholipid bilayer membranes [23, 24]. Some agents inhibit pathogens by disrupting intracellular processes without disrupting cell walls and plasma membranes; for example, sublancin, a glycosylated peptide produced by *B. subtilis* 168, shows antibacterial activity

against methicillin-resistant *Staphylococcus aureus* by disrupting the central dogma [25, 26]. In this regard, *B. subtilis* probiotics are promising antibiotic substitutes with remarkable antimicrobial properties, highlighting their potential as a novel strategy to overcome microbial infections [27]. For example, supplementation with *B. subtilis* PB6 in *E. coli*-infected broilers reduced the mortality rate from 14% to 8%, while antibiotic administration reduced it to 6% [28]. Similarly, compared to negative controls, supplementation with *B. subtilis* DSM 32315 and Nasarin reduced mortality by 6.5% and 9.1%, respectively [29]. *B. subtilis* has also been shown to reduce *Salmonella typhimurium* infection in cell culture studies, in addition to mucosa-associated lymphoid tissue (MALT) healing [30].

2.2 Immunostimulatory effect

B. subtilis stimulates both specific and non-specific immunity, thereby improving protection against pathogens. *Bacillus* spores have been shown to stimulate the immune system by oral administration in numerous studies in humans and animal models. *B. subtilis* spores induce specific humoral and cell-mediated immune responses. The development of both innate and adaptive immune responses in the host is significantly influenced by the interaction between *B. subtilis* spores and macrophages [5]. Studies on macrophages and *B. subtilis* spore co-cultures showed that *B. subtilis* significantly increased the secretion of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β), IL-6, IL-8 and IL-12 [31–33]. Lefevre et al. [34] demonstrated that supplementation of elderly humans with *B. subtilis* CU1 significantly increased fecal and salivary secretory IgA concentrations compared to placebo. Administration of *B. subtilis* MBTU PBBM1 spores to mouse models resulted in T lymphocyte proliferation and accumulation of IgG and IgA antibodies, indicating the potential of *B. subtilis* MBTU PBBM1 spores to enhance both humoral and cellular immunity in mice [35]. Bai et al. [36] showed that administration of *B. subtilis* fmbJ to broilers significantly increased serum IgG and IgA levels. In addition, a study by Li et al. [37] in growing-finishing pigs showed that *B. subtilis* increased serum IgG levels, suggesting that supplementation with *B. subtilis* significantly stimulates the immune response. A study in a rabbit model showed that the combination of *Bacteroides fragilis* and *B. subtilis* had a positive effect on the development of gut-associated lymphoid tissue (GALT) and the diversification of the pre-immune antibody repertoire. Furthermore, two stress responses controlled by Spo0A, including sporulation and TapA (formerly YqxM) secretion, were found to be necessary for GALT development in *B. subtilis* [38].

2.3 Maintenance of intestinal homeostasis

Probiotic *B. subtilis* strains have shown promising results in supporting gastrointestinal health and exhibiting anti-inflammatory properties [5, 6]. Patch et al. [39] found that *B. subtilis* supplementation significantly improved symptoms of constipation, indigestion and dyspepsia compared to the placebo control group. Further research has shown that stool quality was significantly improved following *B. subtilis* supplementation, while stool frequency per day and abdominal noises were significantly reduced [40, 41]. In addition, anti-inflammatory immune cell populations increased in response to *ex vivo* lipopolysaccharide stimulation of peripheral blood mononuclear cells [42]. Studies in piglets and growing-finishing

pigs showed that *B. subtilis* supplementation improved the efficiency of digestion and metabolism of nutrients and had antioxidant effects. *B. subtilis* was shown to have a positive effect on intestinal histomorphology and its supplementation could promote a more favorable mucosal structure and larger absorptive areas of the luminal villi, thus promoting better intestinal development [37, 43, 44]. This finding suggested the reason for the improved digestive efficiency [37]. It was also reported that administration of *B. subtilis* to pigs increased serum superoxide dismutase levels, and after administration of *B. subtilis* fmbJ, reactive oxygen species (ROS) levels in liver mitochondria of broilers were significantly decreased, and the expression of antioxidant enzyme genes including nuclear factor erythroid 2-related factor 2 (Nrf2), haem oxygenase 1 (HO-1), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were improved. Irritable bowel syndrome and inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, have been shown to be associated with oxidative stress and may benefit from antioxidant effects that help maintain and restore intestinal function [45]. Studies in broilers showed that supplementation with *B. subtilis* increased goblet cells in the intestinal tract by 19% and reduced the severity of inflammatory cell infiltration in the jejunum [46, 47]. Research in mice showed that histological damage caused by IBD was reduced by the ingestion of *B. subtilis*, which upregulated the expression of tight junction proteins and suppressed cytokine expression to mitigate intestinal epithelial damage [48]. Furthermore, competence and sporulation factor (CSF) synthesized by *B. subtilis* was found to activate the p38 MAP kinase and protein kinase B (Akt) survival pathways in intestinal epithelial cells, preventing oxidant-induced intestinal epithelial cell injury and loss of barrier function after its OCTN2-mediated transport [49]. CSF also reduced intestinal inflammation and improved survival in mice with lethal colitis [50].

2.4 Maintenance of gut microbiota

B. subtilis administration has been shown to influence the gut microbiota profile by stimulating the growth of normal gut flora and by competitively eliminating pathogens [51]. Studies conducted in broilers showed that *B. subtilis* promoted the presence of beneficial bacteria in the gut such as *Enterococcus*, *Lactobacillus* spp., *Bifidobacterium* spp., *Firmicutes*, *Christensenellaceae*, *Caulobacteraceae*, *Ruminococcus*, *Lachnoclostridium*, and *Anaerostipes* while reducing *Vampirovibrio*, *Escherichia/Shigella*, *Parabacteroides*, *Clostridium*, *Coliforms*, *Campylobacter*, *Bacteroidetes*, and *E. coli* [52–58]. It has also been shown that adherence of *Salmonella enteritidis*, *Listeria monocytogenes*, and *Escherichia coli* pathogens to the HT-29 cell line was inhibited by *B. subtilis* P223 [16]. Furthermore, the *B. subtilis*-treated model organism *Bombyx mori* L. had a higher abundance of *Enterococcus* compared to the control group [59]. Studies in pigs and piglets exhibited that *B. subtilis* supplementation increased the numbers of *Lactobacilli*, *Leucobacter* and *Cupriavidus*, *Coprococcus*, and *Bifidobacterium*, as well as colonic *Succiniclasticum* cells the numbers of *Clostridium perfringens* and *E. coli* [43, 54, 60, 61]. Other studies have demonstrated that *B. subtilis* favors the intestinal colonization of some beneficial bacteria, including *Lactobacillus*, *Leucobacter*, *Bifidobacteria*, *Megasphaera*, *Coprococcus*, *Gemmatimonadete*, *Acidobacteria*, *Ruminococcaceae*_UCG-013 cc, and *Prevotella* [43, 62, 63]. Administration of *B. subtilis* has been shown to alter the microbial composition and optimize the structure of the gut microbiome, contributing to its beneficial effects as a probiotic [37, 43, 44, 63].

3. Applications of probiotic *B. subtilis*

3.1 For humans

Human studies have demonstrated the potential of *B. subtilis* probiotics to effectively treat various intestinal infections and diseases, including antibiotic-associated diarrhea, ulcerative colitis, acute intestinal infections, irritable bowel syndrome, and other infectious pathologies [5, 6]. In addition, probiotic *B. subtilis* has been shown to be effective in the treatment of immunodeficiency [64], Alzheimer's disease [65] and Parkinson's disease [66]. *B. subtilis*-derived probiotics have been shown to increase human longevity by slowing the progression of age-related diseases [67]. In addition, *B. subtilis* supplementation has been beneficial in reducing risk factors associated with cardiovascular disease by lowering cholesterol levels in the human bloodstream [68, 69]. Furthermore, the use of *B. subtilis* natto DG101 in conjunction with appropriate medication has become a promising and innovative intervention in the fight against the global diabetes epidemic [70].

B. subtilis has also shown promise as an adjunctive treatment for *Helicobacter pylori* eradication therapy and dysbacteriosis in neonates and children [5, 6]. Given its ability to favorably alter the gastrointestinal microflora, *B. subtilis* probiotics are being considered as a daily supplement to support overall gut and digestive health in individuals. Probiotic strains of *B. subtilis* have been shown to have beneficial effects on GI health, including improvements in constipation [39], GI quality of life [71], body composition [72–74], stool regularity [40, 41], GI discomfort [75], bloating and belching [76], and microbiome composition [41, 77]. Additional benefits have been observed in glucose metabolism [73], amino acid, purine and vitamin B metabolism [59], GI hormonal response [68, 78], anti-inflammatory activity [42], lipid metabolism [68, 72], endothelial function [68], bone density [79] and immune response [34].

In recent years, engineered probiotics have gained considerable attention as a live therapy to maintain healthy gut microbiota and treat microbial infections. *Lactobacillus rhamnosus* GG (LGG) is a widely studied probiotic for use in ulcerative colitis [80], diarrhea [81] and atopic dermatitis [82]. A major secreted protein, p75, enhances its probiotic activity by maintaining intestinal epithelial cell proliferation and survival by stimulating activation of Akt, suppressing cytokine-induced epithelial cell apoptosis, increasing cell proliferation and reducing tumor necrosis factor (TNF)-induced colonic epithelial damage [83, 84]. However, maintaining the viability of these probiotics during food processing and in the gut remains a challenge [85, 86]. A recent study anchored the p75 protein from *L. rhamnosus* GG to *B. subtilis* via CotG, demonstrating its antibacterial activity against the foodborne pathogen *Listeria monocytogenes* [27]. Researchers have also investigated *B. subtilis* probiotics as a vaccine delivery system. These spore probiotics enhance immune responses by presenting antigens in the gut [87]. For example, researchers have proposed *B. subtilis* spores expressing *H. pylori* urease B (UreB) protein on their surface as a promising oral vaccine against *H. pylori* infection. In experimental mouse models, oral administration of UreB-expressing recombinant *B. subtilis* spores resulted in a significant humoral immune response. Furthermore, oral administration of these engineered spores reduced *H. pylori* load in infected mice by 84% compared to the control group [88]. Several studies have highlighted the importance of surface display of antigens on *B. subtilis* probiotics, such as the C fragment of tetanus toxin (TTFC) [89], the tegument protein of *Clonorchis sinensis* [90] and the SARS-CoV-2 spike protein [91]. These

studies have shown that oral administration of these engineered *B. subtilis* probiotics induces robust immune responses.

Researchers are exploring novel approaches to promote gut health, modify metabolic pathways and improve overall health through the development of recombinant *B. subtilis* probiotic candidates, paving the way for a new era in the treatment of infectious diseases and metabolic disorders. For example, a recent study evaluated the efficacy of a novel recombinant *B. subtilis* SP-64 strain expressing human alcohol dehydrogenase-4 (ADH4) in mice to treat diet-induced metabolic disorders such as obesity, fatty liver disease and type 2 diabetes [1]. Long-term ethanol production in the gastrointestinal (GI) system can damage the gut microbiota and cause organ dysfunction. It is necessary to reduce ethanol exposure to internal organs and increase ADH4 activity, which is necessary for alcohol metabolism [92]. In the study, increased rates of ethanol removal were achieved with the customized *B. subtilis*. Treatment with this customized probiotic significantly reduced weight gain in mice, promoted glucose utilization and prevented pancreatic damage from a high-fat diet [1]. A recent study investigated a lactate oxidase-expressing recombinant *B. subtilis* as a therapeutic probiotic candidate for metabolic disorders associated with elevated blood lactate levels [93]. Anaerobic glycolysis can lead to elevated lactate levels associated with diseases such as cancer, diabetes and mitochondrial dysfunction [94–96]. Traditional techniques for treating elevated lactate levels, such as small molecule inhibitors or enzyme replacement, have limited clinical efficacy [97–99]. In this study, lactate oxidase (LOX)-expressing recombinant *B. subtilis* probiotic candidates were orally administered to the mouse models to improve the rate of removal of elevated blood lactate levels and combat associated metabolic diseases. Oral administration of these LOX-expressing recombinant spore-based *B. subtilis* probiotics significantly reduced elevated blood lactate levels in the animal models without affecting the immune system, liver or gut microbiome [93].

3.2 For animals

Livestock production has expanded significantly to meet the needs of a growing global population, with antibiotics being used as growth promoters to improve feed conversion rates [100–102]. However, this has led to the development of antibiotic-resistant pathogenic bacteria that pose a threat to human health and the economy [103, 104]. The rise of antimicrobial resistance has reduced effective treatments, reduced animal productivity and increased the risk of infectious diseases [105]. The use of antibiotic growth promoters as feed additives has been prohibited in many countries due to regulatory pressure and consumer demand [106]. Alternatives such as probiotics, feed enzymes, prebiotic oligosaccharides, synbiotics, essential oils and immunostimulants have gained interest to maintain current animal production metrics [107]. As a promising probiotic alternative, *B. subtilis* strains have long been used as feed additives to promote animal growth and prevent disease [108]. Its ability to be produced in the form of endospores allows it to rapidly reactivate in the upper intestine to secrete proteases, facilitating the breakdown of complex carbohydrates in plant diets [109–111]. *B. subtilis* produces antimicrobial agents that fight intestinal pathogens and improve intestinal health and food absorption [19–24, 47, 51].

Animal feed production processes typically require high temperatures, and spore-producing bacteria are well suited to these conditions. In the production of functional animal feeds, the ability to sporulate, grow under both aerobic and anaerobic conditions, and survive inside and outside of humans and animals makes the *B. subtilis*

strains one of the most versatile and attractive probiotic bacteria [7, 107]. Several studies have evaluated the effects of *B. subtilis* probiotics on broiler growth performance compared to antibiotics. Lee et al. [112] found that broilers supplemented with *B. subtilis* had higher body weights than those supplemented with 60 mg/kg salinomycin. Teo and Tan [28] showed that the administration of *B. subtilis* PB6 to *E. coli*-infected broilers improved the average daily gain from day 21 to 41 by 7.5% compared to the administration of a combination of 16.7 mg/kg bacitracin zinc and 3.3 mg/kg colistin sulfate. In addition, recombinant *B. subtilis* probiotics in Eimeria maxima-infected broilers significantly improved gut integrity, enhanced innate immunity, and improved growth performance in young chickens [113]. In addition, a study proposed *Clostridium perfringens* alpha toxoid expressing recombinant *B. subtilis* as a promising vaccine for the prevention of necrotic enteritis in poultry [114]. *B. subtilis* spore probiotics expressing a truncated Newcastle disease virus haemagglutinin-neuraminidase protein and a *B. subtilis* strain expressing the duck hepatitis A virus type 1 VP1 protein significantly induced strong systemic and mucosal immune responses in mice, highlighting the potential of *B. subtilis* probiotics as an immunization agent for poultry [115, 116]. The *B. subtilis* probiotic strain was also genetically modified to detoxify poultry feed by inserting the dioxin degradation system of *Sphingomonas wittichii*. It significantly reduced dioxin levels, demonstrating its potential as a feed additive to detoxify poultry diets and provide safe consumption for chickens [117].

Newborn piglets have a sterile gut that eventually develops their characteristic microbiome through interaction with the sow and surrounding environment. The periods immediately following birth and post-weaning represent the most vulnerable stages in a pig's lifecycle, where the elimination of antimicrobial substances from feed could impact mortality rates [118, 119]. A recombinant *B. subtilis* probiotic expressing antimicrobial peptide was developed to address these issues in swine production. Tests on the constructed recombinant probiotics *in vitro* exhibited antimicrobial activity against *E. coli*, *S. typhimurium*, *Haemophilus parasuis*, and *S. aureus*. Furthermore, administration of these engineered probiotics in weaned piglets significantly enhanced the growth and reduced the incidence of diarrhea from enteropathogenic *E. coli* challenge compared to non-supplemented piglets [120].

Porcine epidemic diarrhea virus (PEDV) is a serious pig disease causing high mortality from diarrhea in piglets [121–123]. Currently, available PEDV vaccines lack efficacy, resulting in inadequate treatment [124, 125]. As *B. subtilis* probiotics are known to improve immune regulation and promote gut health in pigs when administered orally [126], engineering *B. subtilis* probiotics to provide immunization against PEDV infection is a promising approach to overcome the current challenges in treating PEDV infection [127]. These probiotics could also be developed as feed additives with enhanced nutritional value. Previous work has shown that tryptophan-excreting *B. subtilis* probiotics can produce tryptophan *in situ* to supplement pig diets, offering the potential benefits of reducing environmental pollution from nitrogen sources and feed costs [128].

Live microbial feed additives, such as probiotics, may not only alter the rumen microbiota but also induce strong host immune responses [129, 130]. Higher feed efficiency and improved milk production have been demonstrated in cows fed *B. subtilis* natto fermentation products, suggesting that *B. subtilis* natto alters rumen fermentation patterns [129, 131]. *Haemonchus contortus* is an important gastrointestinal parasite that infects sheep, causing blood loss and anemia. *H. contortus* infection affects the gastrointestinal microbial population, inhibiting protein digestion and growth performance in infected animals [132, 133]. A recent study showed that HcGAPDH

protein from *H. contortus* expressing a recombinant probiotic *B. subtilis* strain induced immune responses in sheep and reduced worm burden [134].

3.3 For plants

Biological products containing live bacterial cells and/or their metabolites have great potential as next-generation methods of crop protection. These technological advances now allow plant breeders to potentially avoid reliance on chemical pesticide and fungicide applications in some cases. *B. subtilis*, in particular, has shown promise as an effective biological control alternative to chemical control [135, 136]. *B. subtilis* is a common plant growth-promoting rhizobacterium (PGPR) that enhances plant development and yield. It protects the plant against stress through biofilm development, lipopeptide production, siderophore secretion and exopolysaccharide release. It acts as an effective denitrifying agent in the agroecosystem and maintains soil health through environmentally sustainable remediation approaches [137]. For example, the use of *B. subtilis* as a biofertilizer in fertigated Tarocco blood orange groves has been shown to improve fruit quality, soil fertility, growth and nutrient uptake, and to reduce the need for chemical fertilizers [138]. Field trials using *B. subtilis* CPA-8 have reduced *Monilinia* spp. plant infections, demonstrating its potential as an environmentally friendly alternative to fungicides [135]. The chitinase from *Bacillus pumilus* was expressed on *B. subtilis* spores and its biopesticidal activity was demonstrated against fungal pathogens such as *Rhizoctonia solani* and *Trichoderma harzianum*, which are major threats to maintaining sustainable agriculture, highlighting the potential of these novel biocontrol agents [139].

4. Clinical trials

Functional gastrointestinal disorders affect 25–40% of the world's population. They are chronic conditions with persistent symptoms, including functional bowel disorders, irritable bowel syndrome, constipation, diarrhea and bloating [39, 140]. Antibiotics are often ineffective or contraindicated for long-term use, whereas probiotics offer health benefits without resistance [141]. A clinical trial investigated the use of probiotic *B. subtilis* (BG01-4TM) in the treatment of functional gastrointestinal disorders and found that BG01-4TM relieved constipation symptoms, suggesting its potential in the treatment of GI dysfunction [39]. A study of 400 adult patients with moderate-to-severe diarrhea-predominant irritable bowel syndrome (IBS-D) found that *B. subtilis* containing Bio-Kult®, a multi-strain probiotic, reduced abdominal pain and improved bowel movements, suggesting potential benefits for IBS-D patients [142]. A clinical trial in 76 healthy subjects found that daily *B. subtilis* BS50 supplementation for 6 weeks improved bloating, belching and flatulence, suggesting a safe approach to gastrointestinal discomfort [76]. A study in 81 healthy adults aged 18–50 years evaluated the tolerability and safety of *B. subtilis* R0179. The study found no adverse effects on general well-being or gastrointestinal function. *B. subtilis* R0179 was well tolerated at doses ranging from 0.1 to 10×10^9 cfu/day [75].

In a recent study, two cases of type 2 diabetes were well controlled with *B. subtilis* natto DG101 probiotics. Probiotics helped control blood glucose and insulin levels in two cases of type 2 diabetes in obese patients. This study shows the potential of *B. subtilis* probiotics in managing type 2 diabetes and in maintaining health and managing disease [70].

5. Future prospects

Recently, *B. subtilis* as a probiotic has gained attention for its applications in skin care. A study in mice showed that a *B. subtilis*-supplemented Pluronic F-127 hydrogel could modulate the skin microbiome, revealing its potential in the treatment of skin dysbiosis-related diseases such as atopic dermatitis, acne, rosacea, and psoriasis [143]. Furthermore, topical application of *B. subtilis*-substituted polyvinyl alcohol (PVA) microparticles effectively reduced the healing time of open wounds in mice, indicating the potential of *B. subtilis* as an effective therapeutic agent for acute or chronic open wounds [144]. Despite its promising potential, there is a lack of scientifically validated clinical data on the efficacy and safety of probiotic *B. subtilis* formulations for the treatment of skin disorders, which could be urgently pursued as future research.

Psychobiotic strains influence central nervous system (CNS)-related functions and behaviors mediated by the gut-brain axis (GBA) through a number of different pathways [145]. There is growing interest among researchers in the field of microbial-based psychopharmaceuticals. An imbalance in the gut microbiota has been linked to a range of health problems, including inflammatory bowel disease, obesity, type 2 diabetes, and even mental health conditions such as autism, Alzheimer's disease (AD), depression and anxiety [146]. In recent years, preclinical and some clinical studies of psychobiotics have yielded encouraging results in the treatment of neurodegenerative and depressive disorders [65, 147]. Bacteria of the genera *Lactobacillus* and *Bifidobacterium* have been most extensively studied for their psychobiotic effects [146, 147]. Conversely, recent studies have shown that *B. subtilis* strains also have promising psychobiotic properties. For example, studies using chickens as a model organism have shown that *B. subtilis* has beneficial effects on growth performance, intestinal microstructure and digestive enzymes, synthesis and release of antimicrobial and antibiotic compounds, immunity and neurotransmitter activities, including serotonin, and animal behavior following various stressors. In addition, *B. subtilis* has the ability to overproduce L-tryptophan, which subsequently increases 5-HT in the hypothalamus, acting as an antidepressant and anti-anxiety agent, relieving nervous tension in mice and reducing aggressive behavior in laying hens [148]. Besides serotonin, *B. subtilis* has the ability to produce gamma-aminobutyric acid (GABA) [149, 150]. GABA is one of the most important inhibitors in the brain, regulating many physiological and psychological processes. Its dysfunction has been implicated in anxiety and depression. It also plays an important role in the prevention of neurological diseases, cancer, type 1 diabetes and immune disorders [151]. Nitric oxide (NO), another neurotransmitter involved in several GI functions, including maintaining vascular tone, modulating immune responses, transmitting nerve impulses, and facilitating GI motility by relaxing intestinal muscles, could be produced by *B. subtilis* [152]. Alzheimer's disease (AD) is a major cause of dementia among diseases of the nervous system. The exact etiology of Alzheimer's disease remains incompletely understood. There is no definitive and effective treatment for the disease. *B. subtilis* NCIB 3610 has been extensively studied for its ability to form well-structured, three-dimensional biofilms *in vitro*, which is one of the many potential pathways by which it exerts a probiotic effect [153]. It has been shown to delay the onset of age-related neurodegenerative and cognitive deficits in a *Caenorhabditis elegans* model of AD [65]. The anti-AD effects of *B. subtilis* are directly related to the ability of the probiotic to synthesize a quorum-sensing peptide—Competence Sporulation Factor (CSF)—and to gut-associated biofilm development. Alpha-synuclein (αSyn), a highly soluble

unfolded protein, has been implicated in Alzheimer's disease and Parkinson's disease. A reduction in DA levels in αSyn has been observed in post-mortem AD brains, which is associated with cognitive decline. Another *B. subtilis* PXN21 has been observed to inhibit, delay and even reverse αSyn aggregation in young and old *C. elegans* models by altering the sphingolipid metabolic pathway. Despite these promising results, scientifically validated research into their psychobiotic effects has been limited. Their effects may vary depending on factors such as strain type, dosage and individual differences. Comprehensive molecular mechanism studies, animal experiments and clinical trials are therefore essential to refine their use [65, 154, 155].

The potential therapeutic efficacy of *B. subtilis* against coronavirus disease (COVID-19) has recently been elucidated [156]. By modulating the immune system, *B. subtilis* appears to help prevent excessive inflammation and thus improve patient outcomes. Preliminary evidence suggests that this bacterium may have an inhibitory effect on viral replication. This observation warrants further investigation, particularly given the lack of clarity regarding its specific activity against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. *B. subtilis* is still being tested as a treatment for patients with COVID-19 [156] and further studies are needed to confirm its potential and understand how it works.

Recent advances in "omics" technologies, including genomics, transcriptomics, proteomics and metabolomics, have significantly improved our understanding of *B. subtilis* at the molecular level. By integrating omic approaches with systems biology studies, researchers have gained insight into the networks and pathways that control the physiology of this model bacterium [157]. Advances in synthetic biology and genome editing techniques such as CRISPR-Cas9 have led to the development of engineered probiotics for various health problems. These probiotics can be used to treat inflammatory bowel disease, cancer and neurological disorders. *B. subtilis*, known for its non-pathogenicity, excellent protein secretion ability, adaptive metabolism and resistance, makes it an ideal candidate for metabolic engineering and synthetic biology to produce live therapeutics for disease treatment [6, 158]. An intriguing example of such applications is the genetically engineered probiotic *Bacillus subtilis* ZB183, which is Food and Drug Administration (FDA)-compliant and has been developed with the ability to reduce alcohol hangover. This product has been marketed by ZBiotics since 2019 [159, 160]. In addition, in 2024, ZBiotics commercialized another engineered probiotic bacterium, ZB423™, to convert sugar into fiber continuously throughout the day. This unique probiotic produces levansucrase, an enzyme that splits sugar into glucose and fructose, and redirects the fructose to form a rare fiber called levan. This enzyme is delivered directly to the gut, maximizing the benefits and delivering the fiber in a way that is preferred by the microbiome [161, 162]. These two recombinant commercial examples represent a significant advance demonstrating the success and safety of engineered *B. subtilis* probiotics, the number of which is expected to increase rapidly in the near future.

6. Conclusions

The *Bacillus* genus of bacteria has recently become the subject of considerable research interest, due to the significant potential health benefits they offer to humans, animals and plants. For humans, *B. subtilis* offers a wide range of health-promoting properties, including improved digestion, stimulation of the growth of natural microflora, circulatory blood homeostasis, stimulation of the immune system, prevention

of intestinal inflammation, and potential protection against pathogenic invaders. Clinical trials have demonstrated the safety and efficacy of *B. subtilis* as a dietary supplement for the treatment of diseases affecting the gastrointestinal (GI) tract and type-II diabetes. In addition, preclinical studies have also yielded promising results for the management of cardiovascular diseases, inflammatory disorders, dermatological diseases, neurodegenerative and depressive conditions, and even SARS-CoV-2 infection.

B. subtilis is being used more in livestock farming because it improves feed efficiency and eliminates the need for antibiotics. This is achieved by improving nutrient digestibility, reducing pathogen load, strengthening mucosal physiology and improving gut immunity and microbiota diversity in various livestock species. Together with these beneficial effects, the capacity of *B. subtilis* to form spores at high temperatures and to survive in low pH environments renders it a robust strain that can be developed into an in-feed probiotic supplement.

The *B. subtilis* species is of great importance in maintaining plant health, promoting plant growth and fighting against pathogens. *B. subtilis* has been demonstrated to be highly effective in combating pathogens, enhancing nutrient availability and enhancing stress tolerance in plants. This highlights its significant potential in shaping the trajectory of sustainable agriculture.

The advent of synthetic biology with the advancement of genome editing techniques such as CRISPR-Cas9 has engendered promising prospects for the customization of *B. subtilis* strains. As a proficient chassis cell, these organisms can be modified to alter their intrinsic properties, thereby facilitating the development of novel solutions for the treatment of a spectrum of human, animal and plant diseases. Given its status as a commensal organism present in humans, animals and plants, its exceptional resilience even in adverse conditions and its designation as a Generally Recognized as Safe (GRAS) organism, engineered *B. subtilis* probiotics are poised to assume an even more pivotal role in sustainable practices and innovative solutions to the challenges of human, animal and plant health in the near future.

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