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Review

The Complex and Changing Genus *Bacillus*: A Diverse Bacterial Powerhouse for Many Applications

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Abstract: For years, the *Bacillus* genus has encompassed a heterogeneous group of individuals whose main common trait was their ability to sporulate in the presence of oxygen. This criterion has been revised, resulting in the reclassification of several species into new genera and to a redefinition of the characteristics of the members of this taxon. Currently, the species of the genus are grouped into the Subtilis clade and the Cereus clade. The former, called *Bacillus* sensu stricto, initially composed of *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens*, has subsequently incorporated new species related to these. The Cereus clade, *Bacillus cereus* sensu lato, consists of pathogenic species (*B. anthracis*, *B. cereus*, and *B. thuringiensis*) as well as others of significance in agriculture and industry. Furthermore, identifying these individuals remains complex, requiring alternatives to 16S rRNA sequencing. The ability to form spores resistant to stressful conditions provides a significant advantage over other genera, with observable differences in sporulation rates and spore structure among different species. Additionally, *Bacillus* spp. are known for their capacity to produce antimicrobial substances, lytic enzymes, and volatile organic compounds, each with diverse applications. Some species are even used as probiotics. This review delves into aspects related to the taxonomy and identification of microorganisms belonging to the genus *Bacillus*, which often present challenges. The aim is to provide a comprehensive overview of the topic. In addition, it highlights the characteristics and applications of the genus, emphasizing its importance in biotechnology and microbiology.

Keywords: *Bacillus* spp.; taxonomy; identification; sporulation; secreted compounds

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1. New Definitions of a Genus with a Large Number of Microorganisms

The genus *Bacillus* belongs to the Bacteria Kingdom, Firmicutes Phylum, *Bacilli* Class, *Bacillales* Order, and *Bacillaceae* Family. It was created in 1872 by Cohn, who renamed *Vibrio subtilis* as *Bacillus subtilis*, which is a type species of this taxonomic group [1]. For years, this taxon consisted of a heterogeneous group of microorganisms. This heterogeneity was mainly due to the lax criteria used in the past when assigning a genus to various species capable of forming spores in the presence of oxygen [2].

The revision of the genus was conducted through studies of 16S rRNA, which allowed for the characterization of phylogenetic groups that were subsequently reclassified. An example of this is the study conducted by Ash et al., who, based on the phylogenetic analysis of the sequences of 51 species within the genus *Bacillus*, identified five clusters [3]. One of these clusters contained the type species, which they called *Bacillus sensu stricto*, while the other four were later reclassified into the genera *Brevibacillus*, *Geobacillus*, *Lysinibacillus*, and *Paenibacillus* [2].

Subsequently, phylogenetic analyses based on the sequences of other genes/proteins began to be conducted. By 2018, the genus consisted of more than 280 species; however,

extensive polyphyly was observed among many of its members, who had little in common [4–6]. Based on phylogenetic analysis, Gupta et al. proposed the reclassification of 17 clades containing species classified as *Bacillus* into new genera: *Alteribacter*, *Ectobacillus*, *Evansella*, *Ferdinandcohnia*, *Gottfriedia*, *Heyndrickxia*, *Lederbergia*, *Litchfieldia*, *Margalitia*, *Niallia*, *Priesti*, *Robertmurraya*, *Rossellomorea*, *Schinkia*, *Siminovitchia*, *Sutcliffiella*, and *Weizmannia* [7].

Additionally, Patel and Gupta used *core* and other conserved proteins to conduct phylogenetic and comparative genetic sequence analyses, defining the members of the Subtilis and Cereus clades as well as six additional genera: *Alkalihalobacillus*, *Cytobacillus*, *Neobacillus*, *Mesobacillus*, *Metabacillus*, and *Peribacillus* [2]. They also proposed that new species added to the genus *Bacillus* should meet the minimum criteria of the Subtilis or Cereus clades and be supported by a phylogenetic tree based on 16S rRNA sequences or by concatenated protein sequences. The "Subtilis clade", which originally consisted of *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens*, has since expanded to include several species related to these members [8]. This group contains the type species and representatives of the *Bacillus* sensu stricto genus. The "Cereus clade" (Cereus group sensu lato) comprises the pathogenic species of the genus, such as *Bacillus anthracis*, which can cause fatal disease; *Bacillus cereus* sensu stricto, a foodborne pathogen; and *Bacillus thuringiensis*, an entomopathogen, along with non-pathogenic species that have significant applications in agriculture and industry.

Additionally, [9] proposed a nomenclatural framework for the *Bacillus cereus* group that includes: genomospecies defined using resolvable clusters obtained at 92.5 ANI; established medically relevant lineages with a formal collection of subspecies names; and the heterogeneity of clinically and industrially significant phenotypes with a formalized and extended collection of biovar terms. This framework delineates seven genomospecies: (I) *Bacillus pseudomycoides*, (II) *Bacillus paramycoides*, (III) *Bacillus mosaicus*, (IV) *Bacillus cereus* sensu stricto, (V) *Bacillus toyonensis*, (VI) *Bacillus mycoides*, (VII) *Bacillus cytotoxicus*, and (VIII) *Bacillus luti*.

Currently, the genus *Bacillus* comprises 435 species and 12 subspecies (with validated publication and correct nomenclature), which are grouped based on 16S rRNA analysis as well as other genes and protein sequences into two distinct clades organized into phylogenetic trees that are not phylogenetically related to each other (List of Prokaryotic names withstanding in Nomenclature, [10]. This genus, subject to constant modifications, has recently validated species such as *B. arachidis*, *B. changyiensis*, *B. dafuensis*, *B. daqingensis*, *B. dicomae*, and *B. basilensis* [11–19].

2. Alternatives in Species Identification

Given the high genetic homology among members of this genus [20] whole-genome sequencing would be ideal for identifying species; however, it is not always feasible. In such cases, sequencing the *rrs* gene that codes for 16S rRNA with subsequent analysis as a sole methodology does not always constitute an adequate tool for the identification of these microorganisms [3,21,22]. It has been shown that 93.93% of *Bacillus* genus members contain multiple copies of the 16S rRNA gene, and 55.32% of 16S alleles are identical to those of other species [20]. Meanwhile, Mohkam et al. combined the results obtained from 16S rRNA sequencing with the analysis of the sequences of the *rpo*B and *rec*A genes, which code for the β subunit of RNA polymerase and a recombinase, respectively, along with the RAPD-PCR technique for the identification of *Bacillus* species, obtaining better results than using 16S rRNA sequencing alone [22]. Regarding the DNA gyrase gene (*gyr*A), Liu et al. developed new primers (*gyr*A3) that offer enhanced identification and typing capabilities compared to those formulated by [23,24] (*gyr*A1 and *gyr*A2) [25]. While *gyr*A1 and *gyr*A2 primarily detect *B. subtilis*, *gyr*A3 is capable of identifying at least 92 *Bacillus* species, including various species from both clades and related taxa. Furthermore, *gyr*A3 enabled more detailed subspecies-level clustering for *B. amyloliquefaciens*, *B. pumilus*, and *B. megaterium*, a level of resolution not achieved for *B. anthracis*. The limitations in species detection are related to the high variability in protein-coding housekeeping gene sequences,

which complicates the development of universal sequencing primers [26]. Recently, Xu et al. designed primers for the gene encoding the elongation factor thermo-unstable (EF-Tu) [27]. These primers were specific to the genus *Bacillus* and were able to successfully differentiate species both in silico and in vitro.

In this regard, strategies have been developed to identify members of both clades. Since the Cereus group consists of pathogenic species as well as others of importance in agriculture and industry, the identification of bacterial species is essential for assessing public health risk and industrial utility [28]. The virulence factors of pathogenic members of the group are mostly encoded on plasmids, which can be lost; moreover, these virulence factors can be shared among species, complicating diagnosis by traditional molecular methods, such as PCR. Porcellato et al. observed that the analysis of the pantoate-beta-alanine ligase (*pan*C) gene sequence has strong discriminatory power for species identification within the *B. cereus* group [29]. In search of a simple and straightforward assay, Ateiah et al. developed a method for detecting unamplified bacterial 16S ribosomal RNA using a DNA nanomachine (DNM) [30]. This approach aims to simplify the analysis of biological RNA samples and offers potential utility for environmental monitoring. Another significant advancement in the identification of members within this group is the methodology proposed by Tourasse et al., who introduced the first core genome multilocus sequence typing (cgMLST) scheme involving 1568 core genes for the entire *B. cereus* group, including *B. cytotoxicus*, which functions as an outgroup [31]. This scheme has been implemented in the PubMLST resource. The new cgMLST system provides unparalleled resolution compared to existing phylogenetic analysis methods for the *B. cereus* group. With regard to whole genome analysis, Chug et al. evaluated several tools used for the identification of *Bacillus thuringiensis* from whole genome sequencing [32]. They found that IDOPS predicted crystal protein production with the highest sensitivity.

In reference to the Subtilis clade, Lim et al. complemented 16S rRNA sequencing with the *ytc*P gene for the identification of *B. subtilis* [33]. Heo et al. suggests that using the *gmk* gene, a highly conserved gene that codes for the enzyme guanylate kinase, as an alternative to differentiate *B. velezensis* from other closely related species [34]. The polymerase chain reaction is used in other species of this genus, such as *B. amyloliquefaciens*, which is based on the gene encoding α-amylase present in this microorganism. However, although there is a PCR method used for its identification, this gene is not always present in all strains belonging to this species [35,36]. On the other hand, Xu et al. emphasize the high specificity of the *gyr*A and *rpo*B primers intra-specifically within the *B. subtilis* group [27].

Regarding proteomic methodologies, mass spectrometry (MALDI-TOF), whether used alone or in combination with other techniques, has been useful for identifying microorganisms from both clades [37–40]. However, sample preparation remains a critical factor. Janiszewska et al. explored the crucial role of sample preparation in the identification of *Bacillus* species using MALDI-TOF [41]. Although they did not find significant differences between the methods used (the ethanol/formic acid extraction method and extended direct transfer involving the analysis of whole bacterial cells treated with formic acid), they determined that the optimal incubation period for accurate identification ranges from 1 to 12 h.

3. Reformulation of the Description of the Genus *Bacillus* spp.

Based on these restructurings, the description of the genus initially made by Cohn has been reformulated. The genus *Bacillus* consists of bacteria with a straight or slightly curved rod morphology, ranging in size from 0.5-2.5 µm in diameter by 1.2–10 µm in lenght, which can occur singly or in chains of varying lengths [42]. Most species belonging to this genus are Gram-positive, with a few exceptions that are Gram-variable [7]. These bacteria are generally motile by peritrichous flagella, except for *B. anthracis* and some strains of *B. cereus*. Capsule formation is observed in some strains under certain conditions, being polysaccharide in nature for *B. mycoides* and *B. pumilus*, while *B. anthracis*, *B. subtilis*, and *B.*

licheniformis produce poly-ɣ-D-glutamic acid capsules [42]. Additionally, *B. anthracis* and some strains of *B. cereus* produce an S-layer [43].

Most species form endospores in response to nutritional or environmental stress, which are resistant to heat, cold, ionizing radiation, desiccation, and many disinfectants [44–46]. The spores can be oval, cylindrical, or ellipsoidal, and even kidney- or banana-shaped in some strains of certain species, with central, subterminal, or terminal localizations, without deforming the bacterial cell body [42].

Regarding their habitat, these microorganisms are ubiquitous and can be isolated from various environments, most commonly soil and plants. However, other sources include food, clinical samples, animal origins, and marine environments.

The genus comprises aerobic bacteria; however, some members are facultative anaerobes. They are not nutritionally demanding. *Bacillus* spp. grows on nutrient agar or peptone media, showing ideal growth at a neutral pH. Nevertheless, some species can grow at a pH of 9, while others can tolerate a pH of 2 [47]. Their growth temperature ranges from 10 to 45 °C, with optimal growth occurring between 30 and 40 °C [7]. Some species can grow in the presence of NaCl concentrations greater than 7% (w/v). Colony morphology is variable, and some species can produce pigments under certain culture conditions. They are chemoorganotrophic microorganisms, metabolizing organic substances such as amino acids, organic acids, and sugars through aerobic and anaerobic respiration or fermentation, depending on the species and environment [47].

4. *Bacillus subtilis*: The Most Studied Species of the Genus

The designation of the species *Bacillus subtilis* refers to a morphological characteristic of this microorganism, as the term "subtilis" is derived from Latin and means fine or thin. This species has been the most extensively studied within the genus *Bacillus*. It was the first Gram-positive microorganism to have its genome sequenced, which consists of a single chromosome of 4.2 Mbp with a G + C content of 43%, containing approximately 4200 genes, of which 253 are essential for its cultivation in the laboratory [48].

Bacillus subtilis is characterized by being Gram-positive bacilli, measuring 0.7–0.8 µm in diameter by 2–3 µm in lenght, which can be found singly or in pairs, rarely in chains. They are motile due to peritrichous flagellation with a low number of flagella [42]. Their vegetative cell wall features glucan of extreme lengths with typical peptide links bound to N-acetylmuramic acid (meso-diaminopimelic acid direct), teichoic and lipoteichoic acids (comprising 60% of the wall), teichuronic acid, and sugar phosphate polymers [49].

The cell wall of the endospores has a different composition, possessing a thin inner layer closely attached to the inner membrane of the forespore (similar in composition to the vegetative cell) and a thick chemically distinct outer layer called the cortex, which is adjacent to the outer membrane of the forespore [49]. Although it does not always produce a visible capsule under laboratory conditions, it has been observed that the *B. subtilis* 168 strain encodes a capsule in its genome that is both polysaccharide and polyglutamic in nature [42].

This species was one of the first to have the process of sporulation studied. Its spores are cylindrical to ellipsoidal, with a central, paracentral, or terminal location and do not deform the bacterial soma. They are easily transported by air, dispersed by wind, and can survive high temperatures, desiccation, UV radiation, and gamma radiation [42,44].

B. subtilis is aerobic, not nutritionally demanding, it has an optimal growth temperature between 28 and 30 °C and can grow in a range of 5 to 55 °C, with a pH range of 5.5 to 8.5. It can grow in the presence of up to 7% NaCl, with some strains tolerating up to 10%. Colony morphology is variable among and within strains, sometimes appearing as a mixed culture. Colonies range from 2 to 4 mm in size, are round to irregular with wavy to fimbriated margins, and have opaque surfaces that can be rough. Generally, they are whitish but can be pigmented in yellow, orange, pink, red, brown, or black. Texture varies, being moist, mucous, membranous, or rough as they dry.

This microorganism has been isolated from various environments, ranging from soil to marine habitats [42]. Due to its frequent presence in dried grass, it was historically referred to as "hay bacillus or grass bacillus". It has also been isolated from compost, moth larvae, flies, plant tissues, leather, poultry waste and feces, paper, cardboard, gemstones, and the surfaces of ancient stone monuments. The ubiquitous nature of *B. subtilis* is attributed not only to spore dispersion, but also to its ability to grow in diverse environments, including soil, plant roots, and within the gastrointestinal tracts of animals [44].

B. subtilis is known for producing a significant number of secondary metabolites, as 4% of its genome is dedicated to their production, as well as enzymes and volatile organic compounds. Some of these compounds are potent inhibitors of fungi and bacteria, allowing *B. subtilis* to compete effectively in natural environments by degrading molecules to incorporate nutrients. These properties have been utilized by various industries, making *B. subtilis* valuable for applications, such as growth promotion, and as a probiotic [44].

5. Sporulation as a Form of Resistance to Adverse Environments

The genus *Bacillus* is characterized by its ability to form spores in response to adverse conditions, which have been shown to possess resistance to heat, desiccation, and radiation [46,50,51]. Particularly, the species *B. subtilis* has been studied as a model for the sporulation process.

The spore possesses a complex structure that differs from the vegetative cell. The synthesis of the various layers that constitute the spore is a gradual process that begins shortly after the stage of septation. It consists of a thin outer layer of glycoprotein nature called the exosporium, present only in some species. In *B. cereus*, *B. anthracis*, and *B. thuringiensis*, the exosporium has hair-like projections [52]. The exosporium contributes to protection against macromolecules, such as hydrolytic enzymes and antibodies, and provides the spore with resistance to oxidative stress produced by macrophages [53]. Beneath this layer are multiple protein layers called the spore coat, which are crucial for resistance to chemicals and show great diversity among individuals in the proteins that constitute it [52,54]. Between these two layers is the interspace [52]. Below the coat is the outer membrane, which acts as a selective permeability barrier. Following this is the cortex, composed of peptidoglycan with unique features, such as loose linkages, and the presence of muramic δ-lactam (MAL). Beneath the cortex lies the spore core, or protoplast, which contains a structure akin to a vegetative cell. This core features a cell wall, a cytoplasmic membrane (spore inner membrane), and cytoplasm, wherein the DNA, ribosomes, and the majority of the spore's enzymes are situated [55]. Within the spore, a characteristic substance is calcium dipicolinate (CaDPA). The spore protoplast contains only 10 to 30% of the water content of the vegetative cell, conferring heat resistance, chemical resistance (e.g., to hydrogen peroxide), and inactivation of certain enzymes present in the spore. Additionally, heat resistance is due to the presence of a functional cortex and DNA-repairing enzymes that operate under extreme heat, such as RecA [52].

Another characteristic of spores is that they have a pH one unit lower than the vegetative cell and specific proteins called small, acid-soluble spore proteins (SASPs) [56]. SASPs bind to DNA, protecting it from radiation, heat, and desiccation, and serve as a source of carbon and energy for the cell during germination. In the case of *B. subtilis*, the presence of CotA, a copper-dependent laccase, has been observed on the outer part of the coat, contributing to protection against UV radiation and peroxide [52].

The time it takes for the sporulation process to develop can vary between species and even between strains. Some microorganisms have high sporulation rates, while others have low rates. [57] studied the sporulation percentage in strains belonging to different species of the genus *Bacillus* and observed better sporulation rates at 72 h in strains of *B. amyloliquefaciens* subsp. *plantarum*, *B. mojavensis*, and *B. subtilis*.

Due to their high resistance, spores have become products of interest in biotechnology. The species *B. subtilis* has been extensively studied. Among its applications, its use as a platform for producing recombinants proteins, including vaccines, vaccine adjuvants, and

the production of nanobodies, as well as platforms for increasing the thermostability of certain enzymes, can be mentioned [58].

6. *Bacillus* spp.: Producer of a Wide Variety of Molecules

Bacillus spp. can produce many antimicrobial substances, lytic enzymes, and volatile organic compounds, providing an alternative to the use of agrochemicals for phytopathogen control [59]. Furthermore, it has been observed to act on crops by secreting exopolysaccharides and siderophores that inhibit the movement of toxic ions and promote water movement in plant tissues, thereby enhancing plant growth [60]. Some compounds produced by species of this genus are used in human medicine, for the production of biomaterials, or industrially for substances such as detergents or enzymes used in the textile and paper industries, such as alpha-amylase. Species like *B. subtilis* have been genetically manipulated to produce substances on a large scale [61].

Additionally, *B. subtilis* plays an important role in various fields, such as food, feed, cosmetics, chemicals, and pharmaceuticals, as a producer of vitamins (B1, B2, B5, B6, B7, and K), hyaluronic acid, and N-acetylglucosamine [61]. Moreover, *B. thuringiensis* is used for entomological control as it produces exotoxins and endotoxins that are toxic to various orders of insects, mites, and even nematodes [62].

6.1. Antimicrobial Substances Produced by Bacillus spp.

It has been observed that *Bacillus* spp. can produce various antimicrobial compounds that vary in their chemical nature, including ribosomal peptides, polyketides, non-ribosomal small peptide molecules, and lipopeptides.

Ribosomal peptides (RPs) are molecules derived from short precursor peptides (100 aa) that mature through post-translational modifications [63]. This group is primarily composed of bacteriocins, along with other enzymes exhibiting antagonistic activity by interfering with quorum sensing (QS) and its interruption, quorum quenching (QQ), or by inducing cell lysis. Bacteriocins are heterogeneous antimicrobial peptides of low molecular weight, exhibiting varying levels and spectra of activity, mechanisms of action, and physicochemical properties. They possess antimicrobial activity against the same bacteria that produced them or closely related bacteria and can be synthesized by both Gram-positive and Gram-negative bacteria [64]. Karpinski classifies bacteriocins produced by Gram-positive bacteria into four groups: class I (lantibiotics) composed of antimicrobial peptides modified post-translationally and containing unusual amino acids; class II heat-stable peptides that do not undergo modifications and lack lanthionine; class III molecules with a molecular weight greater than 30 kDa; and class IV bacteriocins that require lipid or carbohydrate residues in their molecule for full activity [64]. Examples of bacteriocins include subtilisin, a widely studied lantibiotic synthesized by *B. subtilis*, which forms pores in the cytoplasmic membrane of Gram-positive bacteria, allowing the efflux of ions and metabolites, leading to membrane potential collapse [65,66]. This alkaline protease is synthesized at the end of the exponential phase and the beginning of the stationary growth phase in response to environmental changes such as osmolarity, carbon levels, and nutrient depletion [67]. Lichenin, a class II bacteriocin produced by strain 26L-10/3RA of *B. licheniformis*, is hydrophobic and oxygen-sensitive, exhibiting antagonistic activity against both Gram-positive and Gram-negative bacteria [68]. Another example is amylocyclin, a class I bacteriocin produced by strain FZB42 of *B. amyloliquefaciens*, which shows antagonistic activity against related Gram-positive bacteria [69].

Polyketides (PKs) are a highly diverse family of natural products synthesized from acyl-CoA precursors, such as malonate and methylmalonate. PKs produced by *Bacillus* spp. can be either PKs themselves or hybrids of PKs and nonribosomal peptides (NRPs) [70]. Three types of polyketides produced by *B. subtilis* are recognized, bacillomycin, difficidin, and macrolactin, all of which exhibit antibacterial activity by inhibiting protein synthesis.

Lipopeptides are chemical compounds of a lipid chain covalently bonded to a peptide structure, imparting lipophilic and hydrophilic properties. Many of these compounds

have demonstrated the ability to inhibit bacteria and fungi. The production of such substances has been documented in *Bacillus* spp. [71]. Recently, [72] isolated the *Bacillus amyloliquefaciens* strain LPB-18 capable of producing fengycins and their isoforms, showing the effective inhibition of *Aspergillus flavus* and *Fusarium oxysporum*. Additionally, this strain exhibited characteristics of a potential probiotic due to its tolerance to acidic conditions and bile salts, making it an excellent candidate for biological strains in agricultural products and animal feed.

6.2. Lytic Enzymes Produced by Bacillus spp.

In human medicine, certain enzymes produced by microorganisms can be used as medication. Regarding the genus *Bacillus*, lipase production has been observed, which can be used to hydrolyze fats in chronic gastrointestinal diseases [73], and amylase, which can be used to hydrolyze starch in pancreatic insufficiencies [74,75].

Furthermore, *Bacillus* spp. produces fibrinolytic enzymes, predominantly proteases of the subtilisin family [76], which are often used for thrombolytic therapy in human medicine. These include subtilisin DFE, a serine protease expressed in strains of *B. subtilis* WB600 [77], proteinases QK-1 and QK-2 derived from *B. subtilis* QK02 [78], subtilisin-proteinase like from *B. subtilis* TP-6, and serine proteinases from *B. pumilus* 7P, among others. Furthermore, there are strains that produce enzymes with anticoagulant properties, such as the aforementioned subtilisin-proteinase-like and glutamyl endopeptidase from *B. pumilus* 7P [79]. These enzymes vary in terms of their molecular weight and specific substrate targets, and they can be encapsulated in nanocapsules to achieve greater stability, facilitating easy oral administration [80]. There are also proteinases that act on amyloid plaques (formed by Aβ peptides of 42 amino acid residues) as those produced in Alzheimer's disease. Such is the case of glutamyl endopeptidase, subtilisin-like proteinase, and metalloproteinase produced by *B. pumilus* 7P, which cleave Aβ peptides resulting in the formation of non-pathogenic peptides that lack the ability to form plaques [79], and nattokinase (or subtilin NAT) from *B. subtilis* natto [81]. The formation of amyloid can also be observed in diseases caused by prions. Enzymes such as keratinase from different strains of *B. licheniformis* have been observed to degrade the protein produced in bovine spongiform encephalopathy (PrPsc) [82,83].

Like other bacterial genera, such as *Pseudomonas*, *Acinetobacter*, and *Serratia*, certain species within the genus *Bacillus* also produce enzymes with anti-biofilm activity. Biofilm is defined as a bacterial community embedded in an exopolysaccharide matrix adhered to an inert or living surface. Such is the case with *B. licheniformis*, where the production of protease B [84], alkalase, which is a serine endopeptidase [85], and the endonuclease *Nuc*B [55,86] has been observed. For its part, *B. amyloliquefaciens* produces neutrase, a protease with activity under neutral conditions [87], and the *B. pumilus* strain 3–19 produces subtilisin-like protease and glutamyl endopeptidase, which degrade the biofilm of *Serratia marcescens* [88].

Similar to other biocontrol organisms, *Bacillus* spp. have been observed to produce lytic enzymes. For example, the BT42 strain produces beta-1,3-glucanases that degrade the cell walls of fungi such as *Colletotrichum gloeosporoides* and *Fusarium oxysporum*, potentially causing cell lysis of these microorganisms [89]. Additionally, species of this genus can produce enzymes involved in quorum quenching (QQ) [59].

Similarly, enzymes produced by *Bacillus* spp. can be used as additives incorporated into animal feed. Commercial formulations have been developed that include phytases, proteases, α-galactosidase, xylanases, and α-amylase, and are used in swine and poultry production [90]. Species such as *B. amyloliquefaciens*, *B. licheniformis*, and *B. subtilis* have been identified as sources of amylases and proteases [91].

6.3. Volatile Compounds Produced by Bacillus spp.

Volatile compounds are metabolites that play an important role in microorganisms associated with plants and soil [92]. These compounds disperse easily, performing essential biological and ecological roles in habitats. Volatile compounds can be involved in biocon-

version reactions, biogeochemical cycles of essential elements (carbon, nitrogen, phosphate, and sulfur), and many metabolic and physiological reactions, such as nitrification and nitrogen mineralization. Additionally, they can serve as communication signals (QS/QQ) or defense mechanisms [93]. The volatile compounds produced by *Bacillus* spp. can be both organic and inorganic.

7. Inorganic Volatile Compounds

The inorganic volatile compounds of microorganisms are primarily by-products of primary metabolism and can be carbonaceous or hydrogenated compounds containing sulfur or nitrogen, such as HCN, H_2S, N_2, NH_3, and NO. It has been observed that *B. licheniformis* is a denitrifying bacterium that generates N_2, which is subsequently utilized by nitrogen-fixing bacteria [93].

8. Volatile Organic Compounds (VOCs)

VOCs are molecules with fewer than 20 carbon atoms, low molecular weight (100 to 500 Da), high vapor pressure, low boiling point, and lipophilic character [93], mostly derived from the oxidation of glucose [94]. Due to their low molecular weight, VOCs can diffuse over long distances through soil and atmosphere [95]. Depending on their chemical structure, organic compounds can be classified into fatty acid derivatives (hydrocarbons, ketones, and alcohols), acids, compounds containing sulfur, compounds containing nitrogen, and terpenes [96]. These compounds have garnered interest due to their antibiotic properties or their ability to enhance plant defenses, such as 2,3-butanediol and acetoin [97]. The production and diffusion of VOCs by soil microorganisms depend on factors such as available nutrients, oxygen levels, temperature, pH, physiological state of the microorganisms, moisture, soil texture, and architecture [93]. Additionally, the reduction in pathogen growth by VOCs might not only be due to toxicity, but also to the alteration in the pathogen transcriptome [95]. The production of a wide spectrum of volatile compounds with antifungal activity has been observed in strains of *B. amyloliquefaciens*, *B. subtilis*, and *B. pumilus* [25,98], as well as in strains of *B. amyloliquefaciens*, *B. pumilus*, and *B. cereus* with varying degrees of antibacterial activity [99].

9. Background on the Use of *Bacillus* spp. as a Probiotic

The definition of a probiotic was revised in 2014 by the Scientific Association for Probiotics and Prebiotics, which established different categories for live microorganisms used in humans [100]. In this context, live microorganisms that, when administered in adequate doses, confer beneficial effects on the health of the consumer were categorized into categories: (i) probiotics present in foods or supplements not used for a specific purpose (referring to strains that belong to a species safe for administration and with sufficient evidence of health benefits for the consumer), (ii) probiotics in foods or supplements with a specific health effect on the consumer, and (iii) probiotics as drugs or biotherapeutic agents, which have a specific effect and are therefore used for the treatment or prevention of diseases and are regulated as medicines [101].

There are several reasons why *Bacillus* spp. has been evaluated as a probiotic. First, some species of the *Bacillus* genus and several products produced by species of this genus are recognized as GRAS (Generally Recognized As Safe) by the Food and Drug Administration (FDA, Silver Spring, MD, USA). This acronym refers to any substance intentionally added to food as an additive and subject to prior review and approval by the FDA unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the intended conditions of use or is exempt from the definition of a food additive (FDA, USA). To date, strains of different species have GRAS status, such as *B. subtilis*, *B. coagulans*, *Bacillus clausii,* and metabolites/enzymes produced by *B. deramificans*, *B. licheniformis*, *B. acidopullulyticus, B. amyloliquefaciens*, *B. thuringiensis*, *B. circulans*, *B. subtilis*, and *B. naganoensis* [102].

Bacillus spp. can be manufactured in spore form, which can survive extreme environmental conditions. This ensures the stability of the product during storage at temperatures above room temperature. Additionally, the spores' resistance to high temperatures enables them to be directly mixed into animal feed, which is pelletized at 80 to 85 °C, and are also capable of surviving stabilization methods used in the production of powdered products, such as freeze-drying or drying, which involve cellular dehydration [39,103]. Spores quickly reach the intestine and secret proteases, lipases, and amylases in the upper intestinal tract after reactivation, which are useful for degrading complex carbohydrates. Previous studies have shown that once ingested, the spores of *Bacillus* spp. can germinate and exert their action in the intestine [104–106].

Furthermore, it has been observed that *B. subtilis* can complete an intestinal life cycle in such a way that its spores, after germinating in the intestine, can grow, increase, and re-sporulate, potentially being mobilized by peristaltic movements and released into the environment with fecal matter. This situation could occur in other species of the *Bacillus* genus [107,108]. Additionally, *Bacillus* spp. can colonize the intestinal tract of mammals [109,110]. The colonization after the germination process has been evaluated in mice, showing that *Bacillus* spp. spores germinate in the jejunum and ileum and colonize the intestine temporarily [111]. Another noteworthy characteristic is its ability to form a biofilm [112,113].

Different species of *Bacillus* genus are used as probiotics in human medicine, administered orally, for the prevention and control of gastrointestinal diseases [114,115]. Recently, Tran et al. examined the efficacy of a nasal spray probiotic composed of *B. subtilis* and *B. clausii* spores to support the treatment of influenza viral infections in pediatric patients, achieving promising results [116].

Additionally, many *Bacillus* strains are used as a probiotic in different animals, such as pigs, broiler chickens, calves, and in aquaculture [117–121]. *B. subtilis* is a microorganism that has been incorporated as an additive to the feed of various animal species to improve their intestinal function, and it has also been observed to have an antagonistic effect on intestinal pathogens [61].

Strains from this genus as potential probiotic are under constant study. Recently, Golnari et al. isolated *Bacillus* spp. strains from various sources, including soil, faeces, and artisanal dairy products [122]. They systematically investigated their probiotic properties and compared them with those of some established commercial *Bacillus* probiotics. They obtained eight isolates, which included *B. subtilis*, *B. amyloliquefaciens*, *B. coagulans*, *B. endophyticus*, *B. pumilus*, *B. licheniformis*, and *B. siamensis*. All these strains met the tested probiotic criteria. In this regard, Xiao et al. studied the fermentation carried out by the *B. subtilis* LK-1 strain in Fu brick tea, identifying a total of 45 VOCs, mainly composed of ketones, hydrocarbons, aldehydes, and alcohols, which enhanced the aroma of the final product [123]. Keong et al. also evaluated various strains of *B. subtilis* and found that *B. subtilis* R0179 performed better in fermenting okara compared to *B. subtilis* CU1, *B. coagulans* IS-2, and *B. coagulans* 123, improving both its nutritional value and flavor [124]. Additionally, Bai et al. studied the fermentative capacity of *B. velezensis* CS1.10S and conducted a comparative study of soy sauce fermentation. They observed that, in the presence of this strain, there was an increase in amino acid nitrogen content, reducing sugars, and volatile flavor compounds, while the total acidity did not change significantly [125].

In conclusion, the *Bacillus* genus is undergoing continuous revision and expansion, with the incorporation of new species and the reclassification of some of its varieties into new genera. Although identifying these microorganisms presents challenges, significant improvements are being made through the development of advanced methodologies and the application of integrated identification strategies. This genus offers a multitude of benefits that make it indispensable in various fields. Its ability to produce spores resistant to heat, UV radiation, and desiccation enables it to survive in adverse environmental conditions, ensuring its persistence and effectiveness as a biofertilizer and biological control agent. Additionally, *Bacillus* spp. generate a wide variety of secondary metabolites with

antimicrobial properties, volatile compounds, and enzymes, which are highly valuable in the pharmaceutical and agrochemical industries. Its application as a probiotic in animal and human nutrition not only enhances digestive health, but also strengthens the immune system, and its use via aerogenic routes has been considered for the treatment of respiratory system diseases. Furthermore, due to its fermentative capacity, its relevance in the food industry is significant. These advantages establish *Bacillus* spp. as a multifaceted and essential microorganism in modern biotechnology, providing sustainable and efficient solutions for agriculture, medicine, and industry.

The high capacity of the *Bacillus* genus to produce metabolites has been key in the decision and search for strategies to identify species. Since the identification of the first species in 1835, the genus has undergone numerous changes. Various classifications have been proposed, including their grouping into five clusters in 1991, the characterization of 280 species in 2018, and the proposals of two clades and seven genomospecies in 2020. Currently, the genus is composed of 435 species and 12 subspecies grouped into two clades with no phylogenetic relationship between them, whose members produce various metabolites for medical and industrial use.

Bacillus is a diverse set of species that is easy to culture, with notable advantages for its conservation and distribution, and a broad productive capacity that makes it a biological factory with unlimited potential.

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