Biotechnological applications of *Bacillus licheniformis*

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ABSTRACT

Bacillus licheniformis is a Gram positive spore-forming bacterial species of high biotechnological interest with numerous present and potential uses, including the production of bioactive com- pounds that are applied in a wide range of fields, such as aquaculture, agriculture, food, bio- medicine, and pharmaceutical industries. Its use as an expression vector for the production of enzymes and other bioproducts is also gaining interest due to the availability of novel genetic manipulation tools. Furthermore, besides its widespread use as a probiotic, other biotechno- logical applications of *B. licheniformis* strains include: bioflocculation, biomineralization, biofuel production, bioremediation, and anti-biofilm activity. Although authorities have approved the use of *B. licheniformis* as a feed additive worldwide due to the absence of toxigenic potential, some probiotics containing this bacterium are considered unsafe due to the possible transfer- ence of antibiotic resistance genes. The wide variability in biological activities and genetic char- acteristics of this species makes it necessary to establish an exact protocol for describing the novel strains, in order to evaluate its biotechnological potential.

KEYWORDS

Bacillus licheniformis; industry; probiotics; expression platform; bioremediation; enzymes

Introduction

Bacillus licheniformis is a Gram-positive, endospore-forming organism that can be easily isolated from soil and plant samples. Moreover, in the last decade, several *B. licheniformis* strains have also been isolated from marine environments [1–3]. In the last years, the genomes of different *B. licheniformis* strains have been sequenced due to the increasing biotechnological interest of this bacterial species [4–7]. A previous study has divided the *B. licheniformis* isolates into two groups regarding the presence of the bacitracin synthetase gene and erythromycin resistance [8]. Trying to further understand the genetic heterogeneity of 53 *B. licheniformis* isolates, the multi-locus sequence analysis of six housekeeping genes showed that the strains clustered in the same group with the type strain ATCC14580/DSM13 seem to be more closely related between them than the strains classified in another group [9]. These results support the fact that at least two different lineages that can be clearly recognized are differentiated genotypically, probably because they have evolved differently, are included in *B. licheniformis* species. However, both lineages include strains with an economic interest as well as pathogenic potential, and no connection was observed with the origin of the isolate [9,10].

Bacillus licheniformis is used in combination with *Saccharomyces cerevisiae* for traditional fermented Korean foods [11,12]. Also, *B. licheniformis* is the most abundant bacteria among the complex microbial community (more than 300 microorganisms) involved in the fermentation process of the Chinese *Maotai* flavor liquor [13]. *B. licheniformis* has been safely used for the industrial production of amylase since 1972 [14] and it is considered to have great industrial potential for large-scale production of exoenzymes, including native and recombinant enzymes [5,15,16]. The use of *B. licheniformis* as a feed additive and for the production of beta-cyclodextrin has already been included in the list of "Qualified Presumption of Safety" (QPS) recommended biological agents of the European Food Safety Authority (EFSA) [17]. Moreover, authorities in the United States and Japan have approved products from recombinant *B. licheniformis* strains [14].

Bacillus licheniformis is generally regarded as a non-pathogenic species due to the absence of invasive traits. Nevertheless, some food-borne diarrheal illnesses, toxin production, infant mortality, and bovine abortion have been reported [18,19]. Moreover, the presence of *B. licheniformis* spores in environmental dust was reported to induce inflammation in the respiratory epithelium [20]. Notwithstanding, most infection recorded

cases were associated with incorrect food preparation, infection of preceding tissue injury, intravenous injection, or catheter implantation [13,21]. Additionally, *B. licheniformis* could be considered to be an aerobic spoilage bacterium in the food industry since a recent study was able to identify its presence in artificially contaminated potato salad and milk samples using a quantitative multiplex PCR (mqPCR) [22].

Biotechnological uses with *B. licheniformis*, such as expression platforms, production of molecules, probiotic, and environmental applications, will be discussed in this review (Figure 1). Since the ability of *Bacillus* to produce hydrogen and polyhydroxyalkanoates (PHA) was discussed previously by Kumar et al. [23] these topics have been excluded from this review.

Trends in *Bacillus licheniformis* publications

On June 15th, 2020, the search in the Scopus and the Web of Knowledge (WOS) databases using the query [KEY("Bacillus licheniformis") OR TITLE("Bacillus licheniformis") OR ABSTRACT("Bacillus licheniformis")] resulted in a total of 5,582 and 8,705 documents (Figure 2), respectively. The first work related to the bacterium *B. licheniformis* was published in 1945 [24]. After the first 20 years with just a few publications per year, a slow increase can be observed until 1990 in the WOS database (Figure 2). Then, interest in this species increased exponentially in the following decades. A high percentage of the work related to *B. licheniformis* in both Scopus (74.05%) and WOS (70.92%) databases were published during the last 20 years (Figure 2), indicating an increasing interest in this species.

During this period, the values showed a continuous increase in the publication rate, and 2019 was the year with the highest number of citations, achieving 351 and 482 documents in Scopus and WOS databases, respectively (Figure 2).

Regarding the type of document published in the Scopus database, the majority of the publications were original articles (5216), followed by reviews (124). In the same way, most of the work found in the WOS

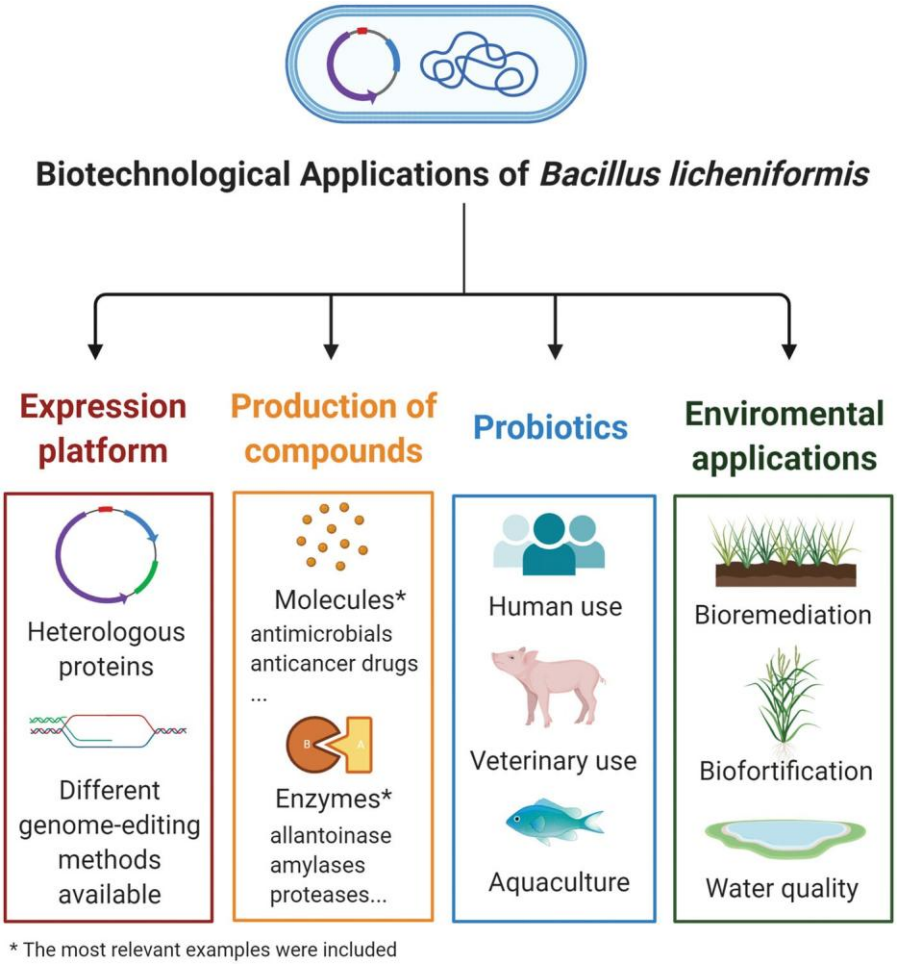

Figure 1. The main biotechnological applications of *Bacillus licheniformis* which are discussed in the present review. Diagram created using Biorender (https://biorender.com/).

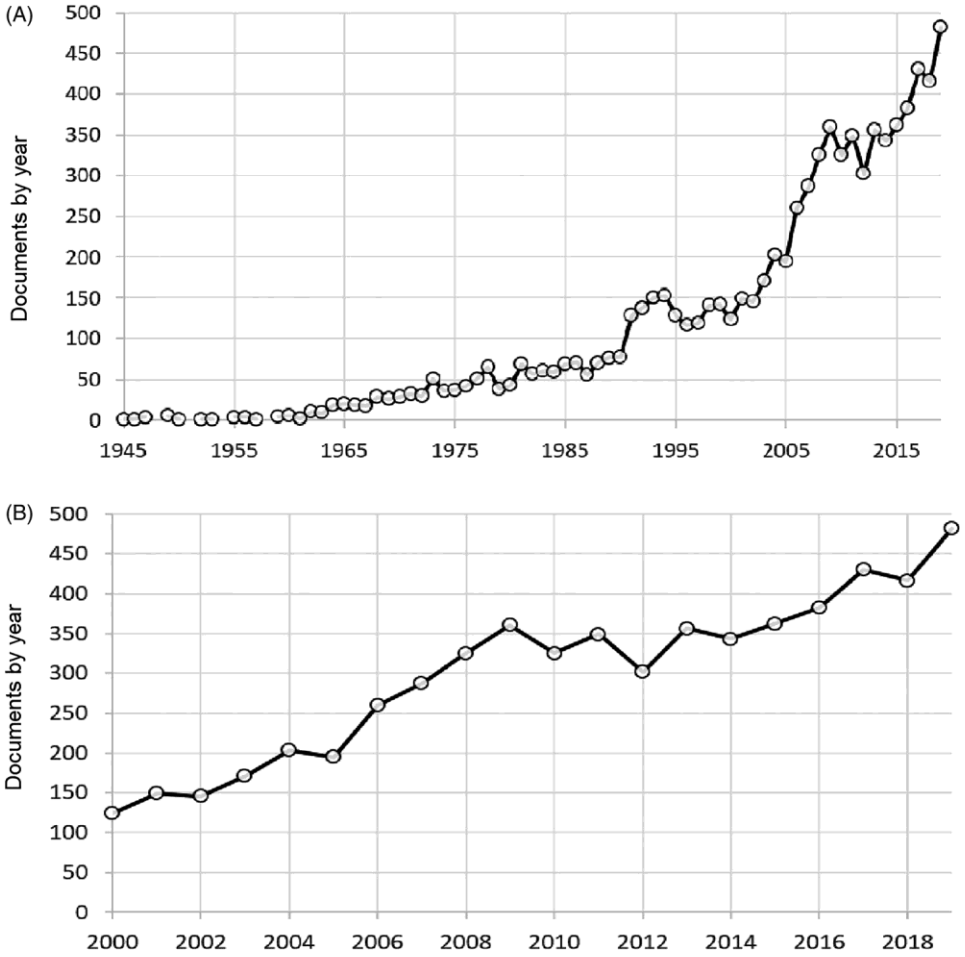

Figure 2. The total number of publications by year (A) and the number of publications between 2000 and 2019 (B). A search from the WOS database was carried out using the search query [KEY ("Bacillus licheniformis") OR TITLE ("Bacillus licheniformis") OR ABSTRACT ("Bacillus licheniformis")].

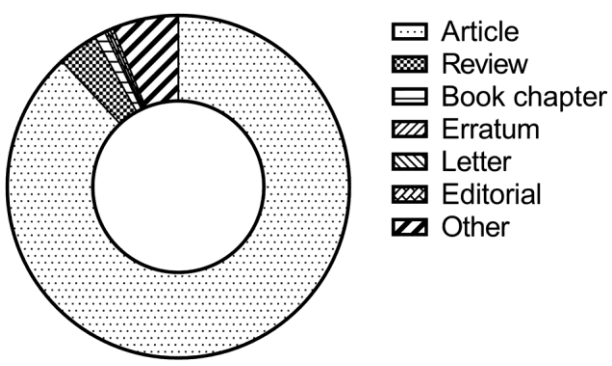

Figure 3. Distribution of document types for *B. licheniformis* until June 2020 according to the WOS database.

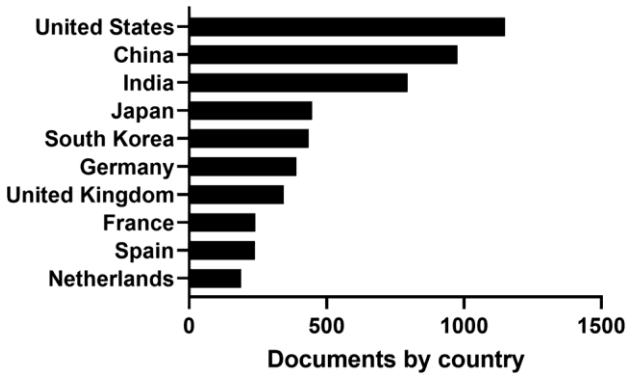

Figure 4. Top 10 countries by number of scientific publications on *B. licheniformis* according to WOS database.

database, based on *B. licheniformis,* were original articles (7,669) and review articles (331) (Figure 3).

In both databases, most documents were produced by the United States (730 and 1149), followed by China (693 and 976) and India (659 and 795) (Figure 4). Other countries such as South Korea, Japan, Germany, United Kingdom, France, and Spain are well-positioned in the top 10 list (Figure 4). The high publication rates in Asian countries could be related to the fact that the use of *Bacillus* probiotics, including *B. lichenifomis,* is widely accepted in Southeast Asia, with numerous products being licensed for different uses.

Bacillus licheniformis as an expression platform

Obtaining desired microbial compounds usually requires the use of industrially relevant microorganisms with the ability to overproduce native or foreign products. In this sense, the application of recombinant DNA technology has played an extraordinary role in the improvement of production yield. *Bacillus* species are commonly employed as host strains for recombinant expression of heterologous proteins, although low transformation rates and no effective genome editing methods have limited its widespread use. *B. licheniformis* WX-02 is a valuable platform host strain for the production of recombinant enzymes such as a-amylase, cyclodextrin glycosyltransferase (CGTase), mannanase and nattokinase as well as for the expression of other bioproducts such as biopolymers [25–31]. Furthermore, different food enzymes produced by genetically modified *B. licheniformis* strains such as a-amylases, b-amylase, maltogenic amylase, acetolactate decarboxylase, and pullulanase were recently evaluated as being safe to use [32–36].

Moreover, a set of shuttle vectors developed by Hentel et al. [37], which allows the genetic modification of *B. licheniformis* and other closely related *Bacilli*, has allowed the overcoming of the traditional difficulties of genetic manipulation with wild-type strains of this species and has extended their applications. Furthermore, a two-fold increase in transformation efficiency was observed when ComI, a competence inhibitor recently identified within this species, was deleted in the industrially-relevant strain *B. licheniformis* ATCC14580/DSM13 [38], further facilitating the use of *B. licheniformis* strains as an expression platform.

Bacillus licheniformis can secrete proteins, especially enzymes, with different applications in large quantities (up to 20–25 g/L; [14]. However, its efficient industrial production required the development of expression systems based on different strategies [39], such as the increase in the expression levels through the amplification of gene copy number [31], optimization of the secretion pathway [40,41], improving RNA and protein stability [27,42], or engineering transcriptional factors [43]. The recombinant strain *B. licheniformis* CBBD302 is the Gram-positive bacteria with the highest production of a-amylase. In this strain, the application of target-directed screening techniques allowed a 26-fold improvement in enzyme production [30]. Also, the integration of multiple copies of a thermophilic a-amylase encoding gene *amyL* into the chromosome of an industrial *B. licheniformis* strain B0204 has been reported to improve its a-amylase production capability [31]. The overexpression of the signal peptide peptidase SppA by inserting expression cassettes into the chromosome of the engineered strain *B. licheniformis* BL10 produced a significant increase in the extracellular production of nattokinase (30%) and a-amylase (67%) [41]. Similarly, the interference with the sRNA *aprA* in the industrial strain *B. licheniformis* DW2 not only caused an enhanced production of the protease subtilisin and the antibiotic bacitracin but also an improvement in the yields of nattokinase (50.53%) and a-amylase (59.81%) [44].

The applications of *B. licheniformis* will surely be expanded thanks to the development of new technologies of genetic manipulation that are recently being explored by the scientific community. In this sense, the transformation of the *B. licheniformis* MW3 strain with a plasmid originally designed for *B. megaterium* allowed the successful production (12–17 mg/L) of the single-chain fragment variable D1.3 scFV, an antibody construct [45]. Recently, a new CRISPR/Cas9 system, which is strictly controlled by xylose, was developed to be used in *B. licheniformis,* showing a useful contribution to improve the success rates of genome editing in this bacterium [46]. Also, the use of CRISPR-Cas9 nickase (Cas9n) was developed in *B. licheniformis* DW2, which allowed improvement of the production of nattokinase by 25.7% [47].

Furthermore, *B. licheniformis* strain 2709 has been genetically modified using a genome-editing method with counter-selectable marker based on a temperature-sensitive plasmid. This method not only optimizes the expression of an alkaline protease gene (*aprE*) but also eliminates the harmful traits related to foaming and extracellular mucopolysaccharide [48]. Two bifunctional expression vectors, based on a free plasmid and an integrational vector, were developed to produce mannanase in a secretory manner, increasing the b-mannanase activity 5–7 times [29]. Recently, the construction of the high-efficient and continuity dual-promoter Pdual3 for enhancing the yield of heterologous protein production on *B. licheniformis* was reported [49].

Production of enzymes and biomolecules

Bacillus spp. are a source of a wide spectrum of enzymatic activities that have been used for decades in the industry. In some cases, the substances obtained from *B. licheniformis* strains presents advantages compared to other bacterial species. Several enzymes with interesting thermoresistant characteristics have been described in *B. licheniformis* strains. Although *B. licheniformis* is a mesophilic bacterium, its a-amylases are

usually more thermostable than those obtained from other microorganisms, including other *Bacillus* species. In this sense, *B. licheniformis* a-amylases presents high optimal temperatures, whose range is between 50–100 ◦C [50–52]. Other *B. licheniformis* enzymes such as b-glucosidase, chitinase, endogluconase, and xylase, presented not only high-temperature stability but also a broad range of pH resistance [53–55]. In this sense, a recombinant phytase from *B. licheniformis* (PhyL) [56] was reported to have an attractive thermostability and high specific activity under a wide range of temperature (4–85 ◦C), and pH and the highest activity among the *Bacillus* phytases described so far [57]. The differential characteristics of these enzymes obtained from *B. licheniformis* seems not to be related to its origin, since the strains were isolated from very different environments such as the rumen or geothermal spring samples [52,54].

Due to the saprophytic style of living of *B. licheniformis*, its secretome is directed to the degradation of a high number of different compounds that includes lipids, polysaccharides, proteins, and other substances. In this sense, this bacterium can produce an extremely high number of different enzymes such as: allantoinase, cellulase, chitinase, cycloglucosyltransferase, dextranase, endo-arabinase (ABNase), glucosidase, glucanase, galactosidase, levanase, lipase, mannanase, pectate lyase, penicillinase, pentosanase or xylanase, and several proteases, including a clostripain-like protease, serine proteases, zinc proteases, glutamyl endopeptidase, metalloproteases and other endo- and exopeptidases [4,5,58–65]. Other novel enzymes with interesting activities have been identified in *B. licheniformis* strains. A new glycine oxidase (BliGO) from *B. licheniformis* strain J33-8 with potential uses in agricultural biotechnology, as well as industrial catalysis, was reported and characterized [58]. *B. licheniformis* SVD1 produces an extracellular multi-enzyme complex called cellulosome that contains different enzymes needed to break down complex plant materials. This cellulosome was not found in the genome of the type strain *B. licheniformis* ATCC14580/DSM13 [59].

Despite the plethora of metabolites with biotechnological interest identified in *B. licheniformis* strains, little is known regarding the effect of culture conditions on the production of these compounds. Interestingly, *B. licheniformis* EI-34-6 produces secondary metabolites in biofilm growth conditions, which were not produced when cells were grown planktonically [66], and therefore any screening process should carefully consider the incubation conditions. The specific culture conditions used, such as the culture media and shaken or not shaken cultures, affects the gene expression and the production of bioactive molecules [67,68]. Hence, the selection of isolation methodology and the culture conditions will have important implications for screening and discovering new biotechnological interesting substances from this species.

Production of enzymes and compounds applied in human health

In the past few years, different enzymes obtained from *B. licheniformis* strains have been tested for their potential biomedicine and pharmaceutical applications. Members of the *Bacillus* group are considered good producers of antimicrobial substances, and *B. licheniformis* has been described as a source of antifungal and antibacterial compounds [69–73] such as the bacteriocin-like peptides lichenin and bacillocin [74–76]. Two lytic enzymes (L27 and L45) from *B. licheniformis* isolates presented activity against cariogenic Streptococci [77]. In agreement with this, some authors suggest that *B. licheniformis* inhibits only Gram-positive pathogens [78]. However, Gram-negative and -positive species' growth inhibition has been also reported [72,79].

Keratinases obtained from different *B. licheniformis* strains were able to modulate scrapie prions proteins either alone [80] or in combination with heat treatment [81]. Different L-asparaginases having low-glutaminase activity from *B. licheniformis* were proposed as potential anticancer drug candidates since they showed cytotoxic activity against several cancerous cell lines [82,83]. Moreover, the L-asparaginase from *B. licheniformis* was demonstrated to be more robust than the commercially available L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi* [82] that have been proved beneficial for chemotherapy treatment of leukemia [84].

The deficiency in Selenium was suggested to increase the risk of suffering certain types of cancer. Therefore, the use of *B. licheniformis* derived biogenic selenium nanoparticles (SeNPs) has been reported as a prevention strategy. These SeNPS were demonstrated to be very useful in inducing prostate cancer cell death while producing lower toxicity than the commercial bioactive selenium supplements in mice models [85].

Another interesting activity described for *B. licheniformis* isolates includes the production of an exopolysaccharide with antiviral and immunoregulatory properties [86] and antimicrobial and larvicidal activity on malaria and Zika virus mosquito vectors [87]. Recently, the silver nanoparticles synthesized by *B. licheniformis* NPS-3 showed excellent antimicrobial activity against critical human pathogens, including

fungi, Gram-positive and -negative bacteria. Moreover, antiviral activity against Bean Yellow Mosaic Virus was also described for these nanoparticles [88].

Resveratrol has numerous potential biological applications as an antioxidant or anticancer agent and presents cardio-protective, anti-estrogenic, anti-inflammatory, and anti-aging properties [89]. Glucosyltranferase from *B. licheniformis* (YjiC) was overexpressed, purified, and incubated with aD-glucose donors to generate resveratrol and other ten glycoside derivatives for the development of new therapeutic agents to treat oxidative stress-induced diseases and provide anti-aging functions [90].

High production of the polysaccharide levan by different *B. licheniformis* strains has been reported [91,92]. Moreover, the levan produced by *B. licheniformis* showed the capability to protect vital tissues such as the kidney, liver, pancreas, and heart from oxidative stress and hyperglycemia in diabetic rats [93]. Nevertheless, whether this is a strain-specific activity remains to be elucidated. Since most studies on *B. licheniformis* are conducted with proprietary and sometimes patented isolates, it is difficult to determine if the enzymes and molecules reported above are a common characteristic of the species or if, on the contrary, they constitute a unique attribute of the particular strain. We propose that the future investigations should include not only the only isolate characterization but also the comparison with the type strain ATCC14580 to clarify that fact.

Probiotic uses

B. licheniformis strains have been included in commercial probiotic products for human health, veterinary application, and aquaculture; however, in most cases, they are used together with other probiotic strains (Table 1). *B. licheniformis* has been extensively used as a probiotic in stockbreeding, acting by promoting growth and as a competitive exclusion agent, stimulating the appetite, facilitating digestion, and increasing nutrient retention and absorption [94–97]. Although the specific mechanisms behind the beneficial effects of *B. licheniformis* probiotics remain mostly unknown, one possible mode of action could involve modulating the intestinal microbiota environment through their ability to decrease pH and improve the intestinal barrier function (Figure 5). The protective action of probiotics could also be explained by their ability to exclude pathogenic microorganisms, stimulate innate immunity, and the plethora of different enzymes produced by *B. licheniformis* species (Figure 5) [69–77,90,93].

The safety of *B. licheniformis* strains as probiotics has been checked in different studies [98–100], and the EFSA has given it the QPS status due to the absence of toxigenic potential [17]. Nevertheless, some probiotics containing this bacterium are considered unsafe by the EU Scientific Committee on Animal Nutrition (SCAN) [101] due to the risk of transferring resistance to erythromycin, as resistance genes were found in a plasmid carried by *B. licheniformis* NCTC13123 [102]. Moreover, other *B. licheniformis* strains have also demonstrated resistance to: clindamycin, kanamycin, streptomycin, ampicillin, cefoxitin, ceftriaxone, cefotaxime, and/or chloramphenicol [102,103]. Therefore, a better understanding of each strain characteristic is necessary to anticipate possible adverse effects of their use as probiotics.

Moreover, since EFSA requires identifying the strains at the species-level to use *Bacillus* for animal nutrition, the use of gyrase A (*gyrA*) and gyrase (*gyrB*) sequences is recommended within the *B. subtilis* group. This is due to the difficulty of taxonomic assignment using standard genetic markers because of the high genetic similarity among *Bacillus* species [104,105]. Therefore, a more in-depth characterization of the used strains and more studies on probiotic-strain safety are required to discard antibiotic resistance genes, toxins, or other substances that may cause harmful effects on the host organisms.

Probiotics for human use

In Southeast Asia, the use of *Bacillus* probiotics for human use is widely accepted and is more extended than in other areas in the world. These products are licensed as prophylactics to prevent rotavirus infections that cause infant diarrhea and to stimulate the immune system [107]. Two of the products licensed as probiotics for humans use are Biosporin® and Primal Defense™ (Table 1), consisting of *B. subtilis* and *B. licheniformis* mixtures. Although some clinical studies have been carried out that demonstrate their safety and efficacy [98,99], most of them have been performed without the rigor of a full clinical trial [107]. Although clinical trials in humans are needed, the therapeutic potential of *B. subtilis* and *B. licheniformis* in the prevention and treatment of periodontitis has been reported in a rat model [109]. Other studies indicate that *B. subtilis* presents higher antagonistic activity against pathogens other than *B. licheniformis* [99].

Several reports have provided evidence of the beneficial effects of *B. licheniformis* for reducing the toxicity of different compounds [100,110–112]. Recently, a

Table 1. Commercial probiotic products containing *B. licheniformis* (modified from [106–108]).

	Commercial product	Company	Comments
Human Use	Biosporin®	1. Biofarm, Dniepropetrovsk, Ukraine 2. Garars, Russia	(1) Mixture 3:1 of *B. licheniformis* 2336 and *B. subtilis* 2335 (2) Versions, including a recombinant form, Subalin
	Primal Defense™	Garden of Life®, Palm Beach, Florida, USA	Mixture including *B. licheniformis*
	MegaSporeBiotic™	Microbiome Labs, Saint Agustine, FL, USA	Blend with spores from five *Bacillus* species (*B. licheniformis, B. indicus, B. subtilis, B. clausii* and *B. coagulans*)
	Prescript-Assist® SBO Probiotic	Genki Health Supplements, GA, USA	28 microbes including B. licheniformis
	Body biotics	Kiki Health, UK	8 bacterial strains including *B. licheniformis*
	Zhengchangsheng®	Aju Pharm., Pyeongtaek, Gyeongi, Korea	*B. licheniformis*
Veterinary Use	AlCare™	Alpharma Inc., Melbourne, Australia	*B. licheniformis* NCTC 13123 spores
	BioGrow®	Provita Eurotech Ltd., Omagh, Northern Ireland, UK	Mixture 1:1 of *B. licheniformis* and *B. subtilis* spores
	BioPlus® 2B	Christian Hansen Hoersholm, Denmark	Mixture 1:1 of *B. licheniformis* DSM5749 and *B. subtilis* DSM5750
	Microsaf®	Phileo by Lesaffre, France	Spore forming bacteria probiotic (B. amyloliquefaciens, B. licheniformis and B. pumilus)
	ZooLac® PROPASTE	ChemVet, DK AS, Denmark	*B. licheniformis* DSM5749, *B. subtilis* DSM5750, *Pedicoccus acidiliactiti, Lactobacillus* sp., and *L. acidophilus* MA64/4A
	Probiotic Soft Chews	ZPaw, FL, USA	*B. subtilis* and *B. licheniformis*
	Vest's Best Probiotic Chews Supplement	Vet's Best	Six bacterial strains including *B. licheniformis*
	Complete Probiotic 4-in-1 for Cats and Dogs	Pet MD™, USA	*B. subtilis, B. licheniformis* and *Lactobacillus acidophilus* bled
	Companions Choice® Prebiotic and Probiotic	Animal-Pro Products, Canada	Mixture of microbes including *B. licheniformis*
	FidoSpore™	Microbiome Labs, Saint Agustine, FL, USA	*B. subtilis, B. licheniformis,* and *Pediococcus acidilactil*
	Probiotics for Pigs	Pangoo Biotech, China	Six bacterial strains including *B. licheniformis*
	B. licheniformis	SinoBios Imp.& Exp., China	*B. licheniformis*
	B. licheniformis	Retron Probiotics, India	*B. licheniformis*
	B. licheniformis	Probioway, Shangai, China	*B. licheniformis*
	Probiotic *Bacillus licheniformis*	Lucky Yinthai Probiotics, Beijin, China	*B. licheniformis*
Aquaculture	BioPlus® 2B	Christian Hansen Hoersholm, Denmark	Mixture 1:1 of *B. licheniformis* DSM5749 and *B. subtilis* DSM5750
	Biostart®	Microbial Solutions, Johannesburg, South Africa, and Advanced Microbial Systems, Shakoppe, MN, USA	Mixture of *B. licheniformis*, *B. megaterium, Paenabacillus polymyxa* and two strains of *B. subtilis*
	BZT® Aquaculture	United Tec Inc. Tulsa, OK, USA	Mixture of *B. licheniformis*, *B. megaterium* and *B. subtilis*
	Efinol PT®	Bentoli, Inc. USA	Mixture of *B. licheniformis, B. subtilis, B. coagulans, Lactobacillus acidophilus,* and other beneficial microorganisms
	Protexin Aquatech®	Probiotics International Ltd, United Kingmdom,	Mixture of *B.licheniformis, B. subtilis, B.polymyxa, B. laterosporus*, and *B. circulans* spores
	Probioway, Shangai, China	*B. licheniformis*	*B. licheniformis*
	Bacillus licheniformis	Vega, Zhejiang, China	*Bacillus licheniformis*
	Probioway, Shangai, China	*B. licheniformis*	*B. licheniformis*
	Pondguard- 100X	Retron Probiotics, India	8 microbial strains including *B. licheniformis*
	BSF-2	Retron Probiotics, India	8 microbial strains including *B. licheniformis*
	BSF-4	Retron Probiotics, India	8 microbial strains including *B. licheniformis*
	Bacitox	Provet Pharma, India	9 microbial strains including *B. licheniformis*
	Everfresh Pro Aqua Probiotics	Blue Weight Biotech, India	4 bacterial strains including *B. licheniformis*
	FeedWale FlocBiotc	FeedWale, India	Mixture of *B. licheniformis, B. megaterium, B. subtilis* and *B. pumilus*
	Sanolife® MIC-F	INVE Technologies nv Dendermonde, Belgium	Mixture of *B. licheniformis, B. subtilis* and *B. pumilus*

study with humans has connected the administration of a *B. licheniformis* probiotic with a lower incidence of dietary endotoxemia [100]. Furthermore, *B. licheniformis* YB9, isolated from a soil sample, showed the capability to degrade the food- and feed-associated mycotoxin deoxynivalenol, inhibiting its damages and repairing the dysbiosis induced in mice [110]. Zhengchangsheng∀demonstrated the reduction in the dextran-sulfate-sodium-induced colitis effects, including weight loss and the disruption of the intestinal barrier integrity [111]. Another commercial probiotic MegaSporeBiotic™ was recently reported to prevent the hepatotoxicity caused by acetaminophen (N-acetyl-p-aminophenol or paracetamol), one of the most used analgesic and antipyretic agents in the world [112]!

Although the mode of action remains unknown, numerous studies evidence the health-beneficial effects of food fermented with *B. licheniformis* in animal models. Previous studies indicate that the antidiabetic properties of soybeans fermented with *B. licheniformis* strains (*chungkookjang*) in rats were equal or better than traditionally made *chungkookjang* [113]. Indeed, improved cognitive function and glucose homeostasis has been reported in diabetic rats with experimental Alzheimer's types of dementia fed with *B. licheniformis* fermented soybeans [12]. The nematode *Caenorhabditis elegans* was used as an *in vivo* animal model to check the beneficial effects of *B. licheniformis* new strains isolated from Korean traditional food (*doenjang, cheongkookjang, kochujang,* and *kanjang*). The probiotic strains identified as *B. licheniformis* stimulated the defense system of *C. elegans* and increased the viability of this nematode when challenged with *S. aureus* [114]. Moreover, *B. licheniformis* probiotic strains modulated its lifespan, enhancing the nematode longevity by influencing 141 genes related with the serotonin pathway [115]. Furthermore, the feeding of rats with yogurt supplemented with *B. licheniformis* fermented pepper juice reduced its body fat accumulation and enhanced lipid metabolism [116].

Probiotics for veterinary use

Numerous studies have suggested beneficial effects of *B. licheniformis* when supplemented as a food additive in pigs, cows, and laying hens [94–97,117]. The inclusion of the *B. licheniformis* strain 1.183 as a feed supplement in Chinese Holstein cows revealed an increase in milk production and milk protein yield, with no significant differences in feed intake, milk fat, or lactose content observed. These beneficial effects have been related to increased fiber digestion in the rumen due to the stimulation of cellulose fermentation by *B. licheniformis* cells [117]. Moreover, a recent study has compared *in vitro* fermentation yields of rice straw and maize stover using different dosages of *B. licheniformis* 10037 and *B. subtilis* 10071. Results indicated that the *B. licheniformis* strain is more suitable for probiotic use than the *B. subtilis* strain, being 0.25×10^7 CFU per 500 mg of the substrate - the most effective dose [118]. In heat-stressed laying hens, the dietary supplementation with *B. licheniformis* caused an improvement of egg production. The addition of this probiotic in the diet was effective for preventing gut morphological alterations caused by heat stress and improved intestinal health and regulated reproductive hormone secretions [96,97,119]. *B. licheniformis* strains DSM17236 and H2 were also reported to prevent necrotic enteritis in broiler chickens [120,121]. When *B. licheniformis* was administered in broilers' drinking water, the productivity also increased compared to the non-treated group [120]. Moreover, an improvement of chicken's nutritional characteristics was recorded in comparison with the non-treated group since lower fat content, an increase of protein and free amino-acid yields, and improvement of sensorial characteristics of the meat was recorded for breast fillets [95].

The efficacy and safety of BioPlus∀2B, a commercial probiotic approved by the EFSA for animal feeding, containing *B. licheniformis* and *B. subtilis* spores (Table 1), has been tested in different animals. Its administration in pigs improved morbidity and mortality caused by diarrhea compared to control groups [122]. In another study, the effect of BioPlus∀2B on young lamb mortality and sheep milk production was tested in late pregnancy and lactation of ewes. Higher milk yield was observed in the probiotic-treated group but did not affect lamb mortality [123]. The feeding with BioPlus∀2B was tested in poultry, increasing the degree of mineralization and the development of bone [124].

Despite the encouraging results obtained with strains of *B. licheniformis* as a food additive in animal breeding, more research is needed to unequivocally attribute these beneficial effects to the probiotic and to understand the mechanisms that induce those effects in order to sustain widespread use of these pro- biotic strains.

Probiotics for aquaculture

Aquaculture is a field in which the use of probiotics is gaining widespread use. Due to the limitations of the use of antibiotics in aquaculture, researchers' efforts have focused on the search for alternatives to control

the proliferation of opportunistic pathogenic bacteria [125]. Numerous studies have shown that *B. licheniformis* can be used against pathogenic fish bacteria, administered as additives in feeds or supplemented into culture water [1,3,20,126–130]. Furthermore, *Bacillus* naturally colonizes shrimps and prawns' intestine, suggesting that it may be used as a biocontrol agent in aquaculture [131]. The efficacy of products with *B. licheniformis* combined with other beneficial microorganisms to fight infections has been demonstrated in aquaculture for crustacean [132–134] and fish species [135]. Different probiotic strains of *B. licheniformis* administrated as a feed additive [126] or included in water [1,127] also enhanced resistance to bacterial infection. An improvement of the resistance against bacterial infection was demonstrated in the common carp (*Cyprinus carpio*) when *B. licheniformis* [129] or secondary metabolites from this species [136] were included in the feed. The dietary administration of *B. licheniformis* in combination with *B. subtilis* and a prebiotic oligosaccharide produced a reduction in the number of gut pathogenic bacteria and an improvement of the innate immunity and disease resistance in shrimp (*Penaeus japonicas*) and triangular bream (*Megalobrama terminalis*) [128,130].

The exact mechanisms of probiotic action it is not well-known. The probiotic character of some *Bacillus* strains could derive from its ability to produce antimicrobial peptides [74,75], anti-biofilm compounds [2,137–139] and to inactivate acyl-homoserine-lactones (AHLs), the quorum sensing (QS) signals controlling virulence in many Gram-negative pathogens [140,141]. Different *Bacillus* species, such as: *B. anthracis* (Ames), *B. cereus*, *B. marcoestinctum*, *B. mycoides*, *B. sonorensis*, *B. circulans* and *B. thuringiensis*, have been reported to degrade QS-signals [142–145]. This activity is produced by AHL-degrading enzymes called lactonases [146], encoded by homologous of the autoinducer inactivation (*aiiA*) gene. A lactonase homologous to AiiA was identified in a *B. licheniformis* DHAB1 strain isolated from healthy Indian white shrimps (*Fenneropanaeus indicus*) that resulted in a reduction in shrimp mortality caused by *V. parahaemolyticus* DAHP1 [3]. Another *B. licheniformis* strain with a lactonase called ytnP was recently been reported to reduce the virulence of the pathogen *A. hydrophila* in zebrafish [147]. Numerous *B. licheniformis* strains isolated from hydrothermal systems also showed the capability to interfere with the QS signal C6-HSL [139]. However, AHL-degrading activity could not be found in the type strain ATCC14580 neither for the short-chain C6-HSL or the long-chain C10-HSL (Muras, et al. unpublished results). All the *B. licheniformis* strains with QS inhibitory activity were isolated from marine or freshwater environments contributing to the idea that AHL-related QS processes could be common in aquatic environments. This supports the idea of their potential use to control diseases in the aquaculture field [67,145,148,149]. Nevertheless, important information is missing regarding the strain-specific multiple mechanisms responsible for probiotic activity in *B. licheniformis,* a field that deserves further research.

Environmental applications

Besides displaying inhibitory activity against bacterial aquaculture pathogens, *B. licheniformis* can also improve water quality due to its capacity to remove nitrogen and phosphorus-based waste accumulation. Since the use of biological agents is dependent on their survival in the environment, *Bacillus* species, as spore-forming bacteria, have clear advantages as bioremediation agents over other bacteria [106]. Because of its denitrification capacity, *B. licheniformis* B003 was reported to positively affect water quality [150]. In another study, *B. licheniformis* BSK-4 was able to significantly decrease nitrite (88.8%), nitrate (72.37%), and total nitrogen (35.95%) in grass carp (*Ctenopharyngodon idellus*) cultures. However, the ammonium concentration increased compared to the control group [151]. Interestingly, the microalga *Chorella vulgaris* and *B. licheniformis* could grow symbiotically, and a combination of both microorganisms achieved better nitrogen (78–88.95%) and total phosphorous (80.28–92%) removal efficiencies, than bacteria or algae alone [152,153]. However, pH control would be essential in this process because this combined system produced a significant acidification of the medium [152].

Among the different bioremediation technologies, using bacteria to improve the nutrient content and reduce pollutants was reported to be a successful management option for contaminated soils [154,155]. The mechanisms for the microbial remediation of combined pollutants includes intracellular and extracellular processes such as: 1) their accumulation by bacterial cells; 2) the uptake and their transformation or degradation; 3) the adsorption onto the microbial surface; 4) the transformation, degradation or immobilization by extracellular compounds, and 5) the redox reaction between the contaminants and the bacteria [154]. *B. licheniformis* has been proved to have positive effects in bioremediation, enhancing the growth of rice in contaminated nickel soils [156], biofortification of wheat with selenium [157], and in phenol degradation studies [158]. Also, a

mercury-removing *B. licheniformis* strain with the ability to remove more than 70% of Hg under optimum conditions has been reported [159].

The ability of *B. licheniformis* to degrade linamarin, a secondary metabolite produced in cassava (*Manihot esculenta* Crantz) processing factories, indicates the potential use of this strain as a detoxicant of cassava wastewaters and a potential application in fertirrigation [160]. A recent study has proposed using shrimp head wastes as a starter for *B. licheniformis* OPL-007 fermentation because the supernatants produced in this fermentation process were rich in bioactive compounds with antioxidant properties and a high nutritional value. Moreover, after protein removal, the substrate could be used as a raw material to produce chitin and its derivatives [161].

Other environmental applications include the production of surfactants. Lichenysin-A, a surfactant obtained from *B. licheniformis* strains R2 and Ali 5, has been proposed as a potential agent for microbial enhanced oil recovery applications since it is stable in extreme environmental conditions such as high temperature, salt concentrations, and a wide range of pH values [162,163]. In the past few years, biomineralization induced by bacterial strains has been proposed as an environmentally friendly method to protect the decayed ornamental stone. *B. licheniformis* AK01 has been proposed as an interesting candidate for this application [164].

Anti-biofilm and anti-fouling compounds

The biofilm growth mode of microorganisms promotes resistance to external insults, including antimicrobial substances [165]. It is estimated that up to 80% of all microbial infections are biofilm-based, being the biofilm infections on medical devices the most critical health hurdles nowadays [166]. Also, biofilm formation is the first step in the development of the biofouling process on submerged surfaces, which is the result of the progressive accumulation of various organism species. Biofouling control is a global economic problem due to its negative impact directly on the shipping industry and indirectly on environmental pollution [167]. Hence, innovative anti-biofilm and anti-biofouling compounds are needed.

Different compounds obtained from *B. licheniformis* strains have been reported to be effective against biofilms formed by a broad range of bacteria [2,137,138,168,169]. The molecules responsible for such activity are very diverse; i.e. a lipopeptide produced by *B. licheniformis* strain V9T14 inhibits biofilm formation by the pathogens *E. coli* and *S. aureus* [137], exopolysaccharides from the *B. licheniformis* strains SP1 and T14 [2,169] and an extracellular protein isolated from the epibiotic tropical strain *B. licheniformis* D1 decreased biofilm formation and disperse pre-formed biofilms of *Candida albicans*, *Pseudomonas aeruginosa* and *B. pumilus* [138]. A new low-cost medium was formulated to optimize the lichenysin production, a biosurfactant with notable anti-adhesion activity, by a *B. licheniformis* strain AL1.1 [170], supporting the idea that the culture conditions can influence the production of bioactive molecules and having critical implications for the screening and discovery of new substances. Regarding anti-biofouling activity, a paint including extracts of culture supernatants of *B. licheniformis* MAI-11-01-CP showed significant inhibition of algal attachment and reduced the surface covered by algal spores [171]. The mechanisms are unknown, although a microbial extracellular DNase (NucB) obtained from the supernatant of cultures of the marine *B. licheniformis* EI-34-6 strain was shown to prevent and disrupt bacterial biofilm formation [168,172]. *B. licheniformis* strain was demonstrated to be a promising approach to control algal blooms since it presents algaecidal activity against the harmful species *Mycrocystis aeruginosa* as well as *M. wesenbergii* and *Phormodium* sp. [173].

Other uses

The production of lipopeptide surfactants by *B. licheniformis* for food preservation application has been recorded [174,175]. In this sense, the inclusion of a partially purified antibacterial peptide (ppABP) from *B. licheniformis* Me1 in active packaging films has proved effective to reduce the growth of bacterial populations, extending the shelf life of food [176]. Recently, the inclusion of ppABP in milk samples showed an increase in the shelf life by 4 days at 28 ± 2 °C [177].

Moreover, it was shown that the inclusion of the calcite precipitating-strain *B. licheniformis* BSKNAU in concrete increases its strength and allows the sealing of the cracks [178]. Possible applications of *B. licheniformis* strains for biofuel production have also been suggested alone [55,179] or in combination with microalgae [180].

Patents

Due to its ability to produce enzymes and bioactive compounds with interesting properties, different *B. licheniformis* strains, as well as methods and applications, have been patented. A search has been carried out at the patent database [181] (www.worldwide.

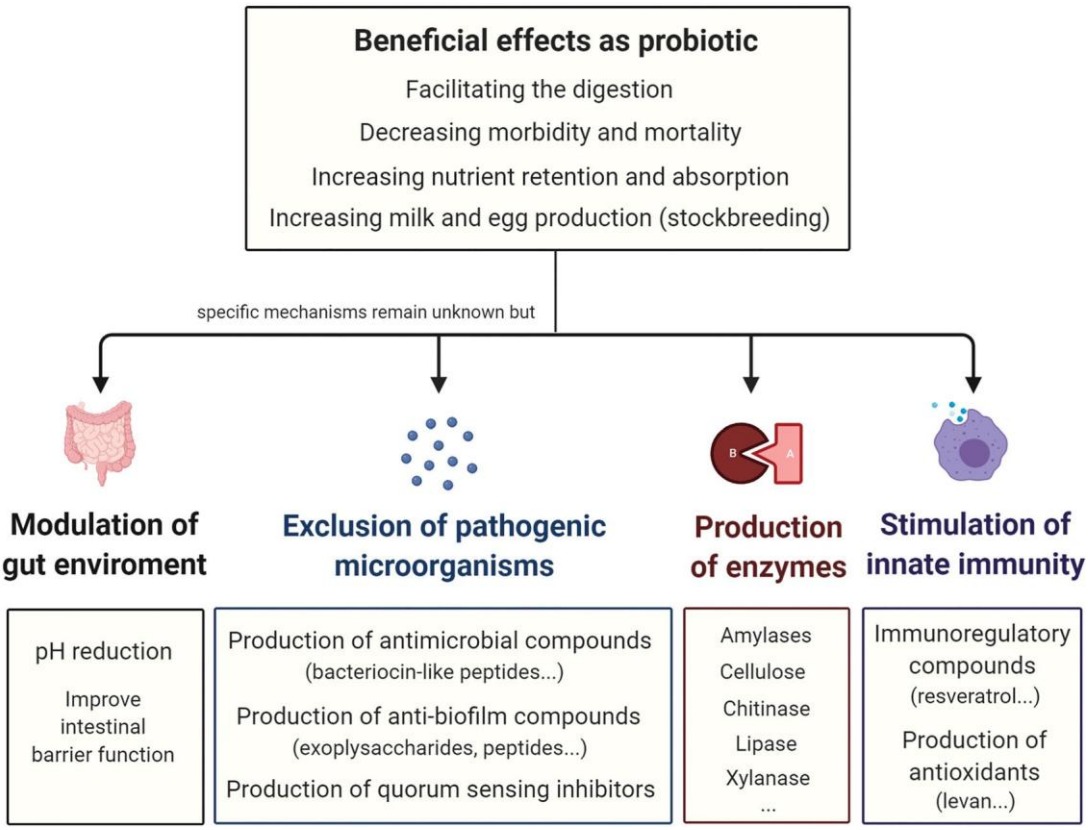

Figure 5. Proposed mechanisms responsible for the probiotic effect of *B. licheniformis*. Diagram created using Biorender (https://biorender.com/).

espacenet.com) revealed 36,063 applications containing the word *Bacillus licheniformis* in the text by April 17th, 2020. Although an increasing number of patents relating to *B. licheniformis* has been observed in the last 20 years (Figure 6), it should be taken into account that in many cases, other bacteria are also usually included in the invention.

If we observe the nationality of the inventors, the results show that most of them came from the USA (26,372 patents), Germany (6645 patents), Denmark (5928), Japan (2220 patents), or China (2118 patents). Similarly, the majority of the applications are performed by institutions or companies in the USA (29,015 patents), in European countries such as Denmark (6993 patens) and Germany (6279 patents), as well as Asian countries as Japan (1976 patents) and South Korea (1648 patents). The USA leads the development of patents related to *B. licheniformis.* In this sense, Monsanto Technology LLC (3524 patents) and Pioneer Hi Bred Int (3161 patents) were the applicants with a higher number of patents, following by Novozymes A.S. (1177 patents).

Regarding the International Patent Classification (IPC), the most common patented uses of *B. licheniformis* strains seem to be related to its use as a host for heterologous protein expression or related to genetic engineering (10,199 patents), being the expression systems specially adapted for plant cells such as plant artificial chromosomes (PACs) being quite common (5766 patents). Besides these industrial applications, a high number of patents are based on preparations for medical or dental purposes with formulations containing peptides (1374 patents), active organic ingredients (1136 patents), or with undetermined constitution (1071 patents). Also, a high number of patents are related to feeding ingredients or processes specially adapted for animals (2212 patents) such as cattle and sheep [182], pigs [183], goats [184], quails [185]. Numerous patents related to the use of *B. licheniformis* in aquaculture have been filled. Most of them have been directed to improving the water quality [186–188], but also probiotics and prebiotic uses have been patented [189–191].

Conclusions

Numerous *B. licheniformis* strains present an excellent potential for large-scale production of bioactive compounds and native enzymes with attractive biotechnological applications. Despite its low transformation

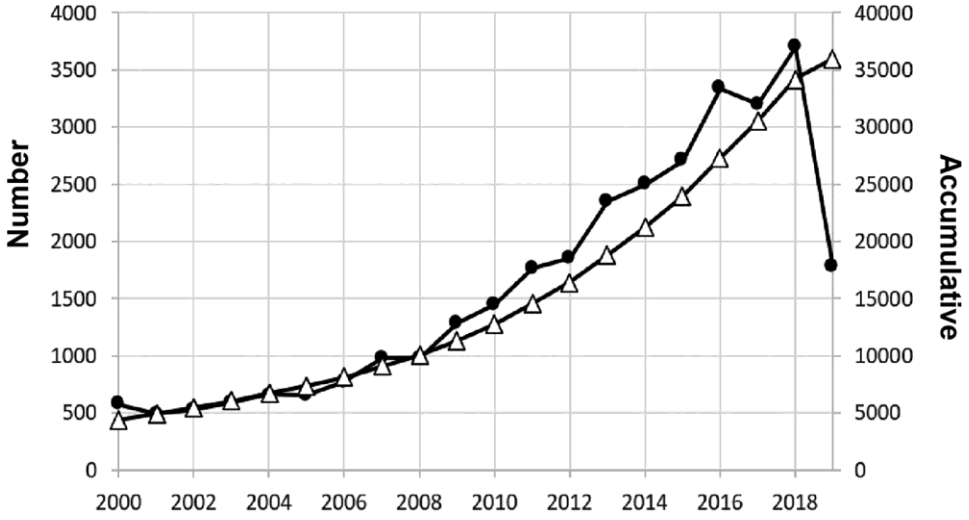

Figure 6. Representation of the Patent applications patented related to *B. licheniformis* in the last 20 years. White triangles (accumulative), black circles (number).

rates, the development of new genetic engineering techniques has allowed *B. licheniformis* to become a valuable platform host strain for the recombinant expression of heterologous enzymes and bioproducts. Some of the compounds produced using *B. licheniformis* strains have shown promising results in improving human health with: immunoregulatory, antimicrobial, antidiabetic properties, and even potential for anticancer drug development. Moreover, the capability to survive the gastric barrier makes *B. licheniformis* more attractive than non-spore forming-bacteria for its use as a food additive and as a probiotic in stockbreeding, not only acting by preventing infections but also as a growth promoter and productivity enhancer. However, some probiotics containing *B. licheniformis* strains are considered unsafe because of their resistance to some antibiotics and the potential risk of transferring resistance to other pathogenic bacteria. Therefore, strains should be carefully checked for antibiotic resistance genes before being applied to a process.

Although *B. licheniformis* is generally regarded as a nonpathogenic species due to the absence of invasive traits, *B. licheniformis* strains have been associated with unknown etiology outbreaks. Therefore, the necessary stages before its application are the accurate taxonomic identification and an in-depth characterization (safety, susceptibility to antimicrobials, and adhesion capability) of the candidate strains. However, the standard identification protocols are not enough within the *B. subtilis* group due to the high genetic similarity. Therefore, the use of gyrase A (gyrA) and gyrase (gyrB) sequences are also recommended. Moreover, continuous safety controls are required for the safe use of this bacterium.

Opinion

The use of *B. licheniformis* has increased continuously in the last two decades due to its numerous potential applications in different fields. Although several *in vitro* and *in vivo* studies have addressed the efficacy of *B. licheniformis*-derived probiotics, some of them are considered unsafe due to its potential capacity to transfer antibiotic resistance, and they have been banned.

Finally, much research is required to elucidate the mechanisms beneath the beneficial effects observed in animal and human health. Additional studies with the rigor of a full clinical trial are needed to confirm the reported health-promoting effects.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

Andrea Muras was financially supported by a predoctoral fellowship from the Conseller´ıa de Cultura, Educacio´n e Ordenacio´n Universitaria, Xunta de Galicia (ED481A-2015/311). This work has been supported by the European project BYEFOULING "Low-toxic cost-efficient environment-friendly antifouling materials" [FP7-OCEAN-2013 612717] and Xunta de Galicia [ED431B 2020/13].

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