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Review

The promises and risks of probiotic *Bacillus* species

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Supplementing the human microbiome with probiotic microorganisms is a proposed solution for civilization syndromes such as dysbiosis and gastrointestinal tract (GIT) disorders. Bimodal probiotic strains of the *Bacillus* genus constitute the microbiota of the human environment, and are typically found in soil, water, a number of non-dairy fermented foods, as well as in human and animal GIT. Probiotic *Bacillus* sp. are Gram positive rods, with the ability of sporulation to survive environmental stress and preparation conditions. *In vitro* models of the human stomach and human studies with probiotic *Bacillus* reveal the mechanisms of its life cycle and sporulation. The *Bacillus* sp. probiotic biofilm introduces biochemical effects such as antimicrobial and enzymatic activity, thus contributing to protection from GIT and other infections. Despite the beneficial activity of *Bacillus* strains belonging to the safety group 1, a number of strains can pose a substantial health risk, carrying genes for various toxins or antibiotic resistance. Commercially available *Bacillus* probiotic preparations include strains from the *subtilis* and other closely related phylogenetic clades. Those intended for oral administration in humans, often encapsulated with appropriate supporting materials, still tend to be mislabeled or poorly characterized. *Bacillus* sp. MALDI-TOF analysis, combined with sequencing of characteristic 16S rRNA or enzyme coding genes, may provide accurate identification. A promising future application of the probiotic *Bacillus* sp. might be the microflora biocontrol in the human body and the closest human environment. Environmental probiotic *Bacillus* species display the potential to support human microflora, however controversies regarding the safety of certain strains is a key factor in their still limited application.

Key words: *Bacillus* sp. for detergents, *Bacillus* sp. probiotic preparation, *Bacillus* sp. probiotic safety, *Bacillus subtilis,* biocontrol, human microbiome, probiotic formulations, spore formers

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Abbreviations: EFSA, European Food Safety Authority; FDA, Food and Drug Administration; GIT, gastrointestinal tract; GRAS, Generally Recognized as Safe; LAB, lactic acid bacteria; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; SP, spore; UGT, urogenital tract; UT, urinary tract; VC, vegetative cell; WFCC, World Federation for Culture Collections

HUMAN MICROBIOME IN THE CONTEXT OF MODERN LIFESTYLE

The human organism comprises approximately 40 trillion cells (approx. 4×10^{13}) with 22 thousand genes, while the microflora present in the whole body and on the surface is estimated to be 100 trillion (10^{14}) microbial cells, described as microbiota, with approximately 2 million metagenome microbial genes (Turnbaugh *et al.*, 2007; Ravel *et al.*, 2014). This overall population of microorganisms has been extensively analyzed since 2007 in the Human Microbiome project, utilizing modern sampling methods at different body locations, DNA/RNA purification techniques, advanced computational technologies with specialized software for fast DNA sequencing, as well as 16S rRNA gene sequence-based analyses, with statistical advances enabling the integration of multi data sets of microbiota colonizing the skin, mouth, esophagus, stomach, vagina, colon, and other body parts. Microbiome studies are crucial for understanding the consequences of modern lifestyle (Schnorr *et al.*, 2016), with the substantial changes of human microflora being the side-effect of accessible antibiotic therapies, presence of antimicrobial factors in the cleaning agents and detergents for everyday cleaning routines, automated washing and dishwashing, abandoned breastfeeding and consumption of highly processed foods.

Since developed countries have greatly decreased human exposure to the microbes, pathogens, commensals, and naturally residing environmental strains, scientists are provoked to ask: aren't we too clean…? The "hygiene hypothesis" (Strachan, 1989) and „microbial deprivation hypothesis" (Bloomfield *et al.*, 2006) state that the rapid rise of atopic, allergy, and asthma disorders (Björkstén, 1994; Björkstén *et al.*, 1999; West *et al.*, 2017; Abreo *et al.*, 2018) in the last 30–40 years may be related to the above-mentioned changes in hygienic and nutritional practices, resulting in the "dysbiosis" state of an organism (Waligora-Dupriet & Butel, 2012). Under these conditions, a growing interest in supplementing and/or supporting the natural and beneficial microflora seems to be a promising natural remedy (Quigley, 2010; Waligora-Dupriet & Butel, 2012).

CONTEMPORARY DEFINITIONS AND HEALTH CLAIMS FOR PROBIOTICS

The beginning of the history of probiotics in the scientific field is associated with the Russian microbiologist Ilya Metchnikoff (1845–1916), the author of the early 20th century work entitled "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace harmful microbes with useful microbes". Metchnikoff associated good health and exceptional longevity of inhabitant groups from Eastern Europe with their orderly consumption of fermented dairy products (Metchnikoff, 1907). According to contemporary authors Havenaar and Huis In't Veld (Havenaar & Huis In't Veld,

1992), a „probiotic is a mono- or mixed culture of live microorganisms which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora". Hence, the emphasis is put on the probiotic microorganisms' positive activity rather than on the route of their administration, extending the modes of possible application. Nevertheless, this proposed definition implies that the term „probiotic" is restricted to products that: 1) contain live microorganisms, e.g. as freeze-dried cells or in a fermented product; 2) improve human or animal health (which can include the promotion of animal growth); 3) cause an effect in the mouth or in the gastrointestinal tract (GIT, e.g. when applied in food or administered capsules, systemic application), in the upper respiratory tract (RT, applied with aerosol, local application), or in the urogenital or urinary tract (UGT or UT, capsules or globules, systemic or local application).

This broadened meaning of probiotics is particularly worth mentioning, as the frequently cited FAO/WHO report from expert consultations (Araya *et al*., 2001) presents a commonly used definition of probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" and subsequently focuses the discussion on "live microorganisms which when consumed in adequate amounts as part of food, confer a health benefit on the host". This FAO/WHO publication is closely related to the theme of that particular meeting, where the main focus was on the scientific background of probiotic lactic acid bacteria present exclusively in food and powdered milk.

Nevertheless, the intrinsic and crucial probiotic feature is the application of species proven to be safe for human or animal health. Many probiotic strains, including the *Bifidobacterium*, *Lactobacillus* and *Bacillus* genera, have been used traditionally for ages in food manufacturing processes and their long history of safe use has promoted them in contemporary biotechnological and health /food related industries. Some of these strains are described as "Generally Recognized As Safe" (GRAS), according to U.S. Food and Drug Administration. The GRAS inventory presents intended conditions of use and laboratory data necessary for the evaluation process, which are given a particular file number (GRN No). The notifier (the producer/seller/company) carries the entire responsibility of ensuring the quality and compliance with legal and regulatory requirements of a notified item. As far as the probiotic strains are concerned, the "*Lactobacillus*" GRAS entry covers 23 positions, spanning in time of closure from Dec. 2005 to Aug. 2018, and lists a number of strains including *Lactobacillus acidophilus NP28, NP 51, La-14, L. lactis, L. casei subsp. rhamnosus GG, L. paracasei subsp. Paracasei, L. reuteri DSM 17938, L. plantarum 299v, Lp 115, L. helveticus* and two *Bifidobacterium* strains. Their intended use is declared to be mainly as an ingredient of food products, e.g. yoghurt, dairy products (GRN 736), powdered infant formulas (GRN 231) or cereals, cheese, and dairy products (GRN 357). Interestingly, the "*Bacillus*" GRAS entry covers as many as 67 positions, recorded from September 1999 to June 2018. Most of these entries relate to biotechnological products, derived from different probiotic *Bacillus* sp. strains. The inventory covers vegetative cells, inactivated cells with thermally killed cell preparations or spore preparations, and biochemical preparations from native and recombinant *Bacillus* sp. strains. Genetically modified *Bacillus* strains carry genes for GRAS enzymes such as acetolactate decarboxylase (GRN 587), β-galactosidase (GRN 649), glucanases (GRN 592), maltohydrolase (GRN 746), phospholipase (GRN 689) or protease (GRN 564), a.o. The present state (by the end of Oct. 2018) of biochemical or biotechnological products, derived from non- modified, probiotic *Bacillus* species and recognized as safe under intended conditions of use, is given in Table 1.

The most important European Union legislative work to date referring to the health and nutritional properties of probiotics in food is still the FAO/WHO Report, which gives precise recommendations towards safety, labeling and characterization of probiotics (Araya *et al*., 2001). Interestingly, in the European Union legal framework, the probiotics are treated as food supplements or additives, and since the impact of probiotic definitions is put on health benefits, intensive legal work has been carried out to develop a system of health claims evalua-

Table 1. Probiotic *Bacillus* species bio-products recognized as safe in GRAS Notice Inventory*.

Bio- products				
Name	Source strain	Intended usage	GRAS notification file number	Date of closure
Enzymes				
β-glucanase	*Bacillus subtilis*	Production of beer and potable alcohol	592	2015
Polygalacturonate lyase (pectate lyase)		Fruit and vegetable purees and concentrates	114	2003
Vegetative cells				
Cells	*Bacillus coagulans*	Water additive for processing of bananas	559; EC**	2015
	Bacillus licheniformis	Water additive for processing of bananas	560; EC	2015
	Bacillus pumilus	Water additive for processing of bananas	561; EC	2015
	Bacillus subtilis	Water additive for processing of bananas	562; EC	2015
Inactivated cells				
Thermally killed cells	*Bacillus coagulans* GBI-30, 6086	Liquid and powdered infant formulas/ Food additive, baked goods, beverages, cereals	670, 725	2017, 2018
Spores	*Bacillus coagulans* GBI-30, 6086	Food additive, baked goods, beverages, cereals, powdered or liquid infant formulas	399, 526, 597, 601, 660, 691	2012, 2015, 2016, 2016, 2017, 2017

*state for Oct 2018, **EC: evaluation ceased for the request of the notifier

tion. The probiotic health benefit of an individual strain or mixed preparation must be assayed *in vivo*, showing a health effect in an appropriate human population. The European Food Safety Authority (EFSA) publishes scientific opinions if the subjected probiotic health claims are consistent with the Regulation on Health Claims (EC No 1924/2006). The prevalent concluding of EFSA reveals that claims have not been established according to regulatory requirements (Salminen & Loveren, 2012). Still, there is no unified and harmonized legal framework, which would indicate detailed conditions to be complied by a strain to be considered as a probiotic.

The probiotic safety responsible bodies are also the United States Food and Drug Administration (FDA), the UK Joint Health Claims Initiative (JHCI), and Japan Food for Specified Health Use (FOSHU) (Elshaghabee *et al.*, 2017).

BIMODAL *BACILLUS* sp. AMONG PROBIOTIC BACTERIA

The globally recognizable group of probiotic bacteria are Lactic Acid Bacteria (LAB), represented by a palette of *Lactobacillus* species (Joshi & Singh, 2012) with *Lactobacillus acidophilus*, *L. bulgaricus*, *L. casei*, *L. fermentum*, *L. helveticuslactis*, *L. plantarum*, *Bifidobacterium lactis*, *B. breve* among others. The LAB species are typically aerotolerant (facultative anaerobic), fermenting, Gram positive, found in and ingested with fermented dairy products like yoghurt, kefir, buttermilk, cheese or fermented vegetables like cabbage or cucumbers. They are known to reside in the human gastrointestinal tract or female genital tract. The forms of application as food supplements are traditionally used lyophilized powders for suspension preparation and encapsulated tablets for oral administration or globules for local application. LABs are able to adhere to gastrointestinal epithelial cells (Castellazzi *et al.*, 2013), thus stabilizing and modulating the inherent gut microbiota, eventually the gut is their primary ecological niche. Other occasionally preferred strains are *Bifidobacterium sp.*, *Streptococcus sp.* or even a few strains of *Enterococcus sp.*

The second group of probiotics is referred to as bimodal or allochtonous, and it includes *Saccharomyces boulardi,* which plays a role in hospital-borne *Clostridium difficile* contamination of the human GIT and in *Escherichia coli* (*E. coli*) infection (Hong *et al.*, 2009). Nevertheless, the group of bimodal probiotics comprises mainly of bacteria belonging to the *Bacillus* genus of Gram positive, rod-shaped, straight cells, ranging from 0.5–2.5×1.2–10 μm in size, often arranged in chains. According to Bergey's Manual of Determinative Microbiology (Holt *et al.*, 2000), the strains belonging to the *Bacillus* genus are chemoorganotrophs, express respiratory or fermentative metabolism, ferment glucose resulting in the production of acid, are positive in the catalase test, and do not reduce sulfates to sulfides. Other biochemical features of the genus, such as nitrate reduction and oxidase production, are variable and dependent on the species.

The *Bacillus* species share the sporulation ability, forming one oval endospore per cell. This is a crucial feature for *Bacillus* sp. to survive environmental stress and harsh conditions of growth, preservation, storage and distribution. Spore formers show vast tolerance and survivability in extreme temperatures, pH (even bile fluids), salt, dehydration or poor nutrition (Holt *et al.*, 2000; Jeżewska-Frąckowiak *et al.*, 2017). Despite being aerobic or aerotolerant (facultative anaerobic), *Bacillus* sp. still can form spores under the air conditions.

The extreme durability of spores is determined by combined factors: the hydrophobic exosporium, consisting of lipids, carbohydrates and proteins; the lowered permeability of cortex- surrounding membrane; the cortex, and the 5–15% dipicolinic acid content in spore dry weight (Bernardeau *et al.*, 2017). Nutrients, lysozyme and cationic surfactants stimulate the exchange of dipicolinic acid and Ca^{2+} ions from the spore core for molecules of water, thus allowing the rehydration of enzymes and spore germination.

HUMAN GIT BEING *BACILLUS* sp. SECOND NATURAL HABITAT

Bacillus sp. probiotic strains comprise the primary, world-wide microbiota of the human environmental habitat, typically found in soil, water, plants, mammals, aquatic animals, insects and other invertebrates (Hong *et al.*, 2009, Table 2). However, in the modern civilized world of food production on industrial scale, consumption of highly processed food and sophisticated hygienic practices, they may be paradoxically considered as probiotics from "unconventional sources". *Bacillus* species are also promising and particularly important for an increasingly growing group of lactose-intolerant individuals. They are typically found in non-dairy fermented products, like a variety of traditional fermented foods, for example Japanese natto (fermented soybeans), Korean kimchi (fermented vegetables, mainly cabbage) and Vietnamese fish sauce (Cutting, 2011), as well as in drinks, juices and on raw and unprocessed fruits and vegetables (Sornplang *et al.*, 2016). *Bacillus sp.* are an alternative to sustain the everyday microbiological balance for human organisms deprived of LAC strain sources.

The bimodal character of *Bacillus sp.* probiotics is revealed when comparing their content in environmental sites and in the human gastrointestinal tract, which is their second true habitat, as proven with spore content analysis, 16S rRNA gene sequencing and RAPD-PCR fingerprinting of soil and samples obtained from GIT and feces (Hong *et al.*, 2009; Plaza-Diaz *et al.*, 2014; Bernardeau *et al.*, 2017). The life cycle of *Bacillus* sp. cells in a host organism consists of vegetative cell (VC) divisions, sporulation resulting in spore (SP) formation, germination followed by a metabolic restart called vegetative outgrowth, proliferation and optional resporulation. All these processes, as well as the overall ratio of endospores to vegetative cells in the transit time, greatly depend on the particular *Bacillus* species, physiological characteristics of the host and the actual location of VCs (for mammals preferably in distal GIT) and SPs (for mammals preferably in upper GIT). The persistence of beneficial *Bacillus* strains in GIT after their withdrawal from the diet is reported to be up to more than 20 days, as demonstrated in animal studies (Bernardeau *et al.*, 2017).

Concerning the palette of specific and non-specific beneficial mechanisms (Table 3, Table 4) pronounced in an organism, including GIT, UT and UGT, *Bacillus* sp. strains should be regarded equally as gut commensals, and not exclusively as soil microorganisms.

BENEFICIAL ACTIVITY OF PROBIOTIC ENVIRONMENTAL *BACILLUS* sp.

There is a spectrum of essential beneficial features, that allow to include certain *Bacillus* sp. into probiotic microorganisms category (Table 3). These model probi-

Table 2. Environmental *Bacillus* spore-formers: selected groups and species, commonly used in probiotic preparations for human and animal use.

Genus *Bacillus* phylogenetic group belonging species	Environmental sources
Bacillus subtilis group	
B. subtilis	soil, water, root of tree, seaweed, larva gut, fermented soybean (natto), kimchi
B. mojavensis	soil of Mojave Desert, soil, river mouth, brackish sediment of the river, spacecraft-associated clean room class ISO 8
B. vallismortis	desert soil in Death Valley, soil, waste water, river, sand dunes
B. amyloliquefaciens	fermented soybean (natto), soil, seaweed, animal feces, camel milk, waste water
B. atrophaeus	soil, air, lake water, decomposed wheat, hay dust, yogurt, fish
B. licheniformis	fermented bean curd, sediment and water from hot spring, larva gut, human excrement
References: Hoa *et al*., 2000; Lyons & Kolter, 2017; Wattiau *et al*., 2001; Elshaghabee *et al*., 2017; Linhuan, 2013; WFCC GCM 2018	
Bacillus pumilus group	
B. altitudinis	soil, lake, mangrove, ore mine, insect gut
B. pumilus	soil, leaf, air conditioner filter, larva gut, seaweed fermented fish paste, rice wine
B. safensis	soil, mangrove water, waste water, river, lake, fermented soybean, molasses waste, fermented yak milk
References: Lyons & Kolter, 2017;Elshaghabee *et al*., 2017; Linhuan, 2013; WFCC GCM 2018	
Bacillus cereus group	
B. mycoides	soil, forest soil, water, pond, sludge, leaf, onion and garlic roots
B. cereus	soil, flower, wood core, mangrove sediment, larva gut, market milk, meal remains, pea soup, javan lori feces
B. toyonensis	mangrove, soil
References: Hoa *et al*., 2000; Lyons & Kolter, 2017; Elshaghabee *et al*., 2017; Linhuan, 2013; Palma *et. al*., 2014; Jiménez *et.al*., 2013A,B; WFCC GCM 2018	
Bacillus alcalophilus group	
B. alcalophilus	feces, human feces, distal human intestine, soil, shore line muds
B. gibsonii	soil, rice, sediment from salt marshes
B. clausii	soil, sediment from salt marshes, clay from grass field,
References: Hoa *et al*., 2000; Elshaghabee *et al*., 2017; Linhuan, 2013; Seckbach, 2012, WFCC GCM 2018	

otic *Bacillus* features, particularly safety and survivability of stress within the host, should be assayed with *in vitro* tests on biochemical models, and *in vivo* tests, before the implementation of a given strain for common use (Papadimitriou *et al*., 2015; Elshaghabee *et al*., 2017).

Probiotic *Bacillus* strains, when applied in the form of health foods and dietary supplements or functional feeds and feed supplements, have numerous documented beneficial effects on humans and animals (Table 4). Although the definition of probiotics highly stresses the "living" form of microorganism, represented by a biofilm of *Bacillus* sp. vegetative cells in the gastrointestinal tract, it is worth noting, that the beneficial qualities are exhibited by the spore forms as well. Biochemical effects induced by the viable *Bacillus* cells include antimicrobial activity of peptide or large protein bacteriocins (subtilin, ericin S, coagulin or megacin) or antibiotics (bacilysin, surfactin) (Abriouel *et al*., 2011; Kadaikunnan *et al*., 2015; Dimkic´ *et al*., 2017; Bernardeau *et al*., 2017), and the activity of secreted enzymes, aiding the host's digestion of nutritional compounds. *Bacillus* biofilm formation supports the host organism against GIT, UGT and UT infections, modulating immune system activity (Table 4). The balancing effect and favorable colonization by *Bacillus* probiotics are sustained even if an administered preparation contains spores (Coppi *et. al*., 1985), or the sporulation occurs in upper parts of the GIT in the stomach, or due to bile activity. *In vitro* models of human GIT with *B. subtilis*, *B. clausii*, *B. pumilus*, *B. cereus*, as well as a recent study on healthy adult human volunteers with *Bacillus subtilis* (Ghelardi *et al*., 2015; Bernardeau *et al*., 2017), revealed germination and outgrowth of spores in the stomach and various gut sections, preferably small intestine. *In vitro* dynamic multicompartmental TIM1 and TIM2 models stimulating the stomach, small and large intestine (Intestinal Models), showed that even 8% germination level of *Bacillus subtilis* provided the sufficient colonization inoculum to decrease *Clostridium* and *Yersinia* strains, at the same time increasing the population of various *Bifidobacterium* species (Hatanaka *et al*., 2012).

Table 3. Essential features for model probiotic *Bacillus* species.

Feature with range of proposed tests
1) Safety (antibiotic resistance[1, 2], production of toxins[3], genetic stability[2]; hemolytic activity[1, 2]).
2) Survivability (survivability in stress conditions e.g. temperature[1], bile concentration[4], pH[4], sporulating activity[1], anaerobic growth[1]).
3) *In vivo* compatibility (activity towards invertebrates[5], rodents[5], mammals[5] and human studies [2,5,6]).
4) Colonization ability (cell surface hydrophobicity[7]; colonization of intestinal mucosa or feces[2, 4,6]).
5) Pathogens biocontrol (bacteriocins production[2,3,7], aggregation with pathogens[5], barrier function of the intestine[5,6], restoring UT, UGT, GIT microflora[6]).
6) Immune system interactions (translocation in the gastrointestinal tract (GIT)[2,5]; anti-inflammatory properties[6]; activating macrophages[3]).
7) Stimulation of gene/protein markers production (markers of: stress response[5], adhesion[5], immune system stimulation[3], nutrients production[3]; enzymes production[2,3,7]).
8) Specific medical conditions improvement (blood/ heart diseases[3,5],anticancer properties[3,5]).

[1]Hoa *et al*., 2000; [2]Sanders *et. al*. 2010; [3]Joshi & Singh, 2012; [4]Sornplang *et al*. 2016; [5]Papadimitriou *et al*., 2015; [6]Reid *et. al*, 2005; [7]Kadaikunnan *et al*., 2015

Table 4. Documented *Bacillus* sp. probiotics targeted applications with detailed examples of their effects in humans and animals.

Targeted application	Example	References
Human products: dietary supplements, health foods		
✓ Food allergy ✓ Intestinal and gastrointestinal disorders	✓ Clinical trials on efficiency and safety of association with simethicone in patients with irritable bowel syndrome ✓ Detected ability to affect immunity and inflammatory genes expression in GIT and the reduction of inflammatory diseases of the gut and liver ✓ Vitamin K production ✓ Anti-cancer properties ✓ Restoration of microflora responsible for the intestinal and systemic immunity	Cutting, 2011; Joshi & Singh, 2012; Castellazzi *et al*., 2013; Urgesi *et al*., 2013; Plaza-Diaz *et al*., 2014; Elshaghabee *et al*., 2017
✓ Gastrointestinal infections ✓ Childhood diarrhea	✓ Bacteriotherapy and bacterioprophylaxis, antimicrobial effect of bacteriocins, against broad spectrum of microbes ✓ Surface biocontrol components for hospital-dedicated cleaning products ✓ blocking a pathogen's signalling system	Hoa *et al*., 2000; Castellazzi *et al*., 2013; Vandini *et al*., 2014; Kadaikunnan *et al*., 2015; Piewngam *et al*., 2018
✓ Urinary tract infections	✓ Reduction of undesired bacteria (*Klebsiella, Proteus, Shigella, Pseudomonas, E. coli*) in the urine of elderly patients with slow/static urine flow ✓ Products for topical and oral treatment	Meroni *et al*., 1983; Cutting, 2011
Animal products: feed supplements, functional feed		
✓ Pathogens in aquaculture ✓ Gastrointestinal disorders in animals	✓ Veterinary growth promoters and exclusion agents ✓ Biological control agents for aquatic environments (shrimps, shellfish) ✓ Activity against bacterial infections (*Salmonella enteritidis,* pathogenic *E. coli*) in poultry	Verschuere *et al*., 2000; La Ragione *et al*., 2001; La Ragione & Woodward, 2001; Thirabunyanon *et al*., 2011; Olmos & Paniagua-Michel, 2014; Yasin *et al*., 2016

CONTROVERSIES ON *BACILLUS* sp. SAFETY

The variety of *Bacillus* species share the prevalent common feature of environmental tolerance, as they can be found in a vast range of habitats over the world (Table 2), and they are usually bio-safe.

Reliable classifications of probiotic *Bacillus* species into groups of posed risks towards healthy adults are available from microorganisms' culture collections from the World Federation for Culture Collections (WFCC GCM, 2018): DSMZ, ATCC, NCIB, BCCM/LMG, a.o., see Collection names under the Table 5. Classification into

Table 5. Examples of probiotic *Bacillus* sp. type strains with numbers in different microorganism collections and GenBank accession numbers for characteristic sequences.

Name of the species	Type strain numbers in different collections*	16S rRNA gene sequence accession number (bp)	Genome sequence accession number (bp)
Bacillus amyloliquefaciens	DSM 7, ATCC 23350	AB006920 (274 bp)	FN597644 (3980199 bp)
Bacillus attrophaeus	DSM 7264, ATCC49337, NRRL-NRS 2123, NBRC 15539	AB 363731 (1475 bp, partial)	GCA_001591925.1 (4158197 bp, contig)
Bacillus cereus	DSM 31, ATCC14579, CCM 2010, LMG6923, NCIB 9373, NCTC 2599	AJ841873.1 (542 bp, partial)	AE016877 (5411809 bp)
Bacillus coagulans	DSM1, ATCC 7050, NCIB 9365, NCTC 10334	DQ297928 (1549 bp, partial)	ALAS00000000 (3018045 bp)
Bacillus pumilus	DSM 27, ATCC7061, NCIB 9369, NCTC 10337, CCM 2144	NR_043242 (1434 bp, partial)	ABRX01000001: ABRX01000016 (3833998 bp)
Bacillus safensis	DSM 19292, ATCC BAA-1126, LMG 26769, NBRC 100820	AF234854 (1434 bp, partial)	ASJD00000000 (3731735 bp, shotgun sequence)
Bacillus subtilis	DSM 10, ATCC 6051, CCM 2216, IAM 12118	LN681568 (1502 bp)	CM000488 (4214598 bp)
Bacillus toyonensis	BCT-7112T, CECT 876T, NCIMB 14858T	NR_121761 (1544 bp, partial)	CP006863 (4940474 bp)
Bacillus vallismortis	DSM 11031, NRRL B-14890, BCRC17183	EF433404 (1468 bp)	AFSH01000070:AFSH01000094 (series of shotgun sequences)

*Collection names: ATCC: American Type Culture Collection; BCCM/LMG: Belgian Bacteria Collection; BCRC Bioresource Collection and Research Center (Chinese Taipei); CCM Czech Collection of Microorganisms; CECT Spanish Type Culture Collection; DSMZ: Deutsche Sammlung von Microorganizmen und Zellkulturen (eng. German Collection of Microorganisms and Cell Cultures); IAM Culture Collection (Japan); JCM Japan Collection of Microorganisms; NBRC Culture Collection (Japan); NCIB/NCIMB: National Collection of Industrial Food and Marine Bacteria (UK); NCTC National Collection of Type Cultures (England); NRRL Agricultural Research Service Culture Collection (USA).

Risk group 1 (e.g. in German TRBA, Technical Rules for Biological Agents) is assigned to Prokaryotes that are unlikely to cause an infectious disease in humans, according to the European Directive (2000/54/EC), while a Biosafety level designated as BSL1 (e.g. in American microorganisms collections) refers to the cultures that are not known to harbor an agent that causes disease in healthy adult humans. The cultures that are designated as Risk group 2 or BSL2 present a moderate risk of infection among healthy adults. The numerous strains of *Bacillus* sp. groups shown in Table 2 are described as BSF1 (ATCC) and RG 1 (DSMZ): *Bacillus subtilis*, *B. amyloliquefaciens*, *B. mojavensis*, *B. vallismortis*, *B. atrophaeus*, all strains mentioned in the *B. pumilus* group, *B. clausii* and *B. alcalophilus* from the *B. alcalophilus* group, *B. toyonensis* from the *B. cereus group, B. mycoides* and *B. coagulans*, a.o.

However, *Bacillus* sp. strains are also well known to produce toxins, such as hemolysins, phospholipases, and other enterotoxins. Traditional microbiological plating and biochemical methods for strains characterizations are time-consuming and lack sensitivity or selectivity. Thus, the determination of strains and toxins is performed on *B. subtilis*, *B. pumilus*, B. *licheniformis*, *B. polymyxa*, *B. thuringiensis* and multiple *B. cereus* strains (ATCC 33018, CA6, CA1, MS1-9, HS23-11) (Gray *et al.*, 2005; Owusu-Kwarteng *et al.*, 2017), using cell cytotoxicity assays, with Ped-2E9-murine hybridoma lymphocytes and CHO-based assays, as well as PCR methods. Hemolysin and lecithinase toxins, emetic toxins, diarrheal toxin, B component (dermonecrotic), EntFM (enterotoxic, induces vascular permeability), CytK (necrotic enteritis) and toxin genes *bceT*, *cytK*, *nheA*, *nheB*, *nheC*, *hblA*, *hblC*, *hblD*, *entFM* are typically found in a number of *B. cereus* strains (Gray *et al.*, 2005; Hwang & Park, 2015).

It is worth mentioning, that available genetic data for *B. cereus* (whole genome sequence from GenBank, 2018; Table 5) show numerous intrinsic similarities with *Bacillus anthracis* and *Bacillus thuringiensis* (Ivanova *et al.*, 2003; Rasko *et al.*, 2005; Palma *et al.*, 2014). *B. anthracis* and *B. weihenstephanesis* are examples of pathogenic *Bacillus* sp., which produce toxins, with different levels of toxicity, posing human or animal-health risk (Riedel, 2005; Żakowska *et al.*, 2012; Elshaghabee *et al.*, 2017; Palma *et al.*, 2014).

Certain severe cases of *B. cereus* – related food poisoning have been reported (Dierick *et al.*, 2005). A spe-

Table 6. List of European, African and American household chemicals, containing probiotic *Bacillus* species.

Cosmetics
body spray, body wash, hand soap, skin cream, skin repair concentrate, toothbrush cleaner
Household chemicals
allergen remover spray, baby bottle washing-up liquid, bathroom and toilet cleaner, cleaning concentrate (general purpose), cleaner (general purpose), dish washing-up liquid, drain cleaner, floor cleaner, laundry detergent concentrate, odor and stain remover, septic tank treatment, water system treatment

cific *B. cereus* strain, detected from human isolates and food remains, caused toxic, severe pulmonary haemorrhage, coma, diffuse bleeding and muscle cramps. Results of PCR amplification, as well as cytotoxicity tests of isolates, confirmed the presence of lecithinase, the beta- hemolytic toxin and heat-stable emetic toxin – cereulide, which was the direct cause of death of poisoning as soon as 13 hours past meal.

Interesting representative of a non-toxigenic and non-pathogenic strain of the *B. cereus* group (Table 2) is *B. toyonensis* (*Bacillus cereus* var. *toyoi*) (Jiménez *et al*., 2013b). It is applied in animal nutrition under the name of Toyocerin® probiotic preparation, with no reported cases of toxicity, since its first authorization in Japan, in 1975. *B. toyonensis* does not produce diarrheal or emetic enterotoxins, thus no enterotoxicity, eye irritation, genotoxicity, acute, subchronic or chronic toxicity were detected at the tested doses, in safety studies including human clinical trials (Williams *et al*., 2009).

Besides toxin production, antibiotic resistance is a crucial factor to be taken into consideration in the matter of probiotic *Bacillus* sp. safety (Gueimonde *et al*., 2013; EFSA Panel on Biological Hazards, 2012). Particularly, the possibility of transferring genes of antibiotic resistance may pose a potential health risk of increasing the presence of antibiotic resistance in bacteria of human/animal organisms. In this context, mobile, extra-chromosomal elements, such as plasmids with *erm*(C) or *tet*(L) genes, coding for macrolide or tetracycline resistance, respectively, and conjugative transposons Tn5397, carrying genes for tetracycline resistance *tet*(M), were reported cases in *Bacillus subtilis*. On the other hand, examples of antibiotic resistance determinants present on the bacterial chromosome, such as *aaD2* (aminoglycoside resistance), *erm*(34) (MLS, macrolides, lincosamides and streptogramines resistance), BCL-1 (β-lactams resistance) and *cat*(Bcl) (chloramphenicol resistance) are found in the *Bacillus clausii* DSM8716 strain, and used as a probiotic supplement for diarrhoea prevention in humans (Gueimonde *et al*., 2013).

Nonetheless, it seems that controversies around *Bacillus* sp. safety are still the crucial factor of their consistently limited application as probiotics.

CHARACTERISTICS OF *BACILLUS* sp. PROBIOTIC PREPARATIONS

The probiotic species of the *Bacillus* genus, based on full length 16S rRNA gene sequences, prevalently belong to the subsequent phylogenetic groups (clades): *Bacillus subtilis* group, *Bacillus pumilus* group and *Bacillus cereus* group (Table 2) (Wattiau *et al*., 2001; Elshaghabee *et al*., 2017; Lyons & Kolter, 2017). *Bacillus thuringiensis*, belonging to the *B. cereus* group (Miller *et al*., 2018), is in turn a biotechnological source of parasporal Cry protein (crystal), used as an agricultural biocontrol agent with insecticide activity (Ben-Dov, 2014; Djenane *et al*., 2017). Other strains, often found in commercial supplements of probiotic preparations for humans or for biotechnology purposes, are *B. clausii* or *B. coagulans*.

Commercially available *Bacillus* sp. probiotic starter cultures and probiotic preparations have diverse microbiological species characteristics. There are numerous reports of applying single species formulations (Hoa *et al*., 2000; Hong *et al*., 2005; Cutting, 2011; Olmos & Paniagua-Michel, 2014; Vandini *et al*., 2014), with *Bacillus subtilis* often being preferred due to being the best studied probiotic. Double strain preparations often utilize the composition of *Bacillus subtilis* with *Bacillus* from the *subtilis* group, namely *B. mojavensis*, *B. vallismortis* (US origin strains), *B. amyloliquefaciens*, *B. atropheus*, *B. lichenifromis* or from other closely related clades such as: the *pumilus* group (*altitudinis*, *pumilus*, *safensis*), the cereus group (*cereus*) or *alcalophilus* group (*clausii*). Species are preferably mixed in a 1:1 ratio (Leuschner, 2006; Leuschner, 2008), although the final composition of a preparation may vary after the storage period due to kin discrimination of *B. subtilis* towards very closely related species such as *B. mojavensis* and strains from the *pumilus* group, and better coexistence with strains from the *cereus* clade (Lyons & Kolter, 2017). The mixtures of several strains are named consortiums (Havenaar & Huis In't Veld, 1992; Hoa *et al*., 2000; Cutting, 2011; Olmos & Paniagua-Michel, 2014; Safitri *et al*., 2015) and may consist of *Bacillus subtilis* group strains mixed with other closely related group strains, as well as *Lactobacillus* strains in case of the oral probiotics (Cutting, 2011).

It is worth noting that despite the advanced microbiological techniques used in biotechnological production processes, many commercially available probiotic preparations, even for oral administration in humans, are still being mislabeled or poorly characterized (Hong *at al*., 2005; Lewis *et al*., 2016, Jeżewska-Frąckowiak *et al*., 2017). According to the Bergey's Manual of Determinative Microbiology (Holt *et al*., 2000), a precise "species differentiation is difficult, because of the large number of representatives and often incomplete descriptions of newly discovered species". The morphology of single colonies of different species on agar media often seems superficially very much alike, making it tricky to differentiate during analytical or diagnostic manipulations (Standards Unit, PHE, 2015; Standards Unit, PHE, 2018). Bacterial colonies of *Bacillus* sp. may differ more under different growth conditions for one species, than between two different species grown simultaneously in the same conditions. This phenomenon also extends to the shape of single cells observed under the microscope. The conditions of nutrient limitation drive the population of *Bacillus subtilis* cells to form a mixed population, where half of the population activates the genetic regulator of sporulation and the second half omits this path. Another example of heterogeneity is the coexistence of single swimming cells with an active factor for motility (σ^D ON), and long chains of cells with motility factor switched off (σ^D OFF) (Kearns & Losick, 2005). The motility features seem to increase with higher temperatures, where mucoid or slimy colonies appear (Berkeley *et al*., 2008).

Difficulties in identification result from biochemical features as well, since akin strains of one *Bacillus* sp. clade, e.g. *subtilis* or *cereus*, may barely differ in the composition of fatty acids sustaining their biological membranes (Roberts *et al*., 1994; Roberts *et al*., 1996), have highly similar genome architectures, with ANI (average nucleotide identity) values app. below 94% and display proteome conservation (Earl *et al*., 2012; Jiménez *et al*., 2013b).

However, the microbiological identity of probiotic preparation genus/species can be investigated by the unique microbial protein spectrum (proteome) mass spectroscopy analysis in MALDI-TOF assays (Azarko & Wendt, 2011; Kosikowska *et al*., 2015), as presented for closely related *Bacillus* species: *Bacillus subtilis*, *Bacillus mojavensis*, *Bacillus vallismortis*, *Bacillus pumilus* residing in the same lyophilized preparation sample (Jeżewska-Frąckowiak *et al*., 2017). Alternatively, a specific determination can be achieved with qualitative PCR reactions, amplifying

specific genome regions (Table 5) characteristic for either a group of probiotic bacteria (Wattiau *et al*., 2001) or a single *Bacillus* species (Ashe *et al*., 2014). Many species from the *Bacillus* genus are known to display high similarity in the conserved regions of 16S rRNA genes, like *Bacillus subtilis* and *Bacillus pumilus* (Berkeley *et al*., 2008). The highly conserved sequences became a taxonomic marker, not a species marker (Wattiau *et al*., 2001). It is possible to design PCR primers differentiating between systematic groups of the *Bacillus genus*, e.g. to distinguish the *B. subtilis* group from *B. subtilis*, *B. pumilus*, *B. atrophaeus*, *B. licheniformis* and *B. amyloliquefaciens*. An even more specific determination is possible when the unique marker of a strain is determined, like endo-β-1,4-glucanase of *B. subtilis* (excluding *B. pumilus, licheniformis, amyloliquefaciens, thuringiensis, megaterium*). In this case, combining the results of two PCR primer sets leads to an accurate determination of group (genus) and species (Ashe *et al*., 2014). The PCR reaction products can, of course, also be further cloned and/or sequenced or mapped by the RFLP technique (Restriction Fragments Length Polymorphism), providing even more accurate and precise data. Nevertheless, rapid and reliable identification of *Bacillus* species is still a challenge due to their very high genome, proteome, and metabolic similarities.

The most thoroughly characterized strains are available under the name of type strains, which according to the International Code of Nomenclature of Prokaryotes (latest version from Parker *et al*., 2015) are regarded as reference points for any detected strains that could belong to that species. Many probiotic *Bacillus* species are available in their type strains version, biochemically and genetically characterized, with additional detailed information regarding cultivation conditions, safety, risk groups and a list of references. Examples of probiotic *Bacillus* sp. type strains, with identity numbers in different microorganism collections and chosen information, covering available genome and 16s rRNA gene nucleotide sequence data with GenBank accession numbers, are given in Table 5 (GenBank, 2018).

INDUSTRIAL FORMS AND STABILIZATION METHODS FOR *BACILLUS* sp. PROBIOTICS

The number of possible strain compositions in probiotics reflects the various forms of preparations developed for each intended application. The common industrial forms of *Bacillus* sp. probiotic preparations are liquid solutions and concentrates or lyophilized powders for resuspension closed in a capsule, vial or pouch.

Traditionally, commercial starter cultures of probiotics and ready-to-use probiotic microbiological preparations are supplied in a liquid form (solution or concentrate) that can be used directly for the purpose, e.g. *B. coagulans* in suspensions (Hu *et al*., 2016), or as a microbiological starter in food production, biological control agent, the component of a biodegrading mixture, or surfactant. The liquid cultures, such as *Bacillus* sp. starter culture, when dedicated for pharmaceutical use in humans or animals, can be further concentrated or preferably also stabilized (Kringelum *et al*., 2000), similar to what was described for *B. coagulans*, *B. licheniformis*, *B. pumilus* and *B. subtilis* for future food infusion (Kirejevas & Kazarjan, 2012). An example of a stabilizing solution for *Bacillus,* but also *Lactobacilli* and *Bifdobacteria*, is vegetable (sunflower seed, olive, maize, soya, lineseed, sesame, rice) or animal (fish) oil with the addition of polysaccharides, such as maltodextrin or inulin (Mantzouridou *et al*., 2012).

The necessity for long-term storage of probiotic preparations, the need for increased stability, as well as transport requirements come along with convenience of use and optimization of the probiotic delivery. These are the crucial considerations and reasons for developing solid form probiotics cultures preservation technologies. The *Bacillus* probiotic spore formers are perfect model microorganisms surviving stabilization methods that generate powder products, like freeze-drying (lyophilization) or drying, which both involve cell dehydration (Goderska, 2012; Martín *et al*., 2015). The present trends in probiotic delivery cover a whole palette of microencapsulation methods, which can significantly increase cell viability during freezing or drying processes (Both *et al*., 2012; Chávarri *et al*., 2012; Martín *et al*., 2015). The inner core of the microcapsule is composed of the bacterium cell or cells and the shell is sustained by a supporting material. Popular supporting materials used in microencapsulation extrusion techniques include alginate solutions (algae derived heteropolysaccharides), whey proteins, pectin, milk or human-like collagen (Chávarri *et al*., 2012; Martín *et al*., 2015). A probiotic *B. coagulans* strain was successfully encapsulated using polysaccharides, chitosan and alginate (Anselmo *et al*., 2016), while the extracellular matrix produced by *B. subtilis* is proposed to serve as a protectant for other probiotic bacteria in complex preparation (Yahav *et al*., 2018). The addition of cryo-protectants such as glucose, maltodextrin, trehalose, skimmed milk powder, whey protein or novel soybean flour additionally increases the survival rates and the activity of intracellular enzymes of the freeze-dried encapsulation probiotics, as demonstrated for *B. subtilis* starter cultures or LAB (Martín *et al*., 2015, Mahidsanan *et al*., 2017).

FUTURE TRENDS FOR PROBIOTIC *BACILLUS* sp. IN MANAGING HAZARDOUS BIOFILMS

Probiotic use in humans is still widely associated with orally administered supplements, with an increasing range of *Bacillus* sp. preparations exemplified by Bacticubtyl® (*B. cereus*, France, Germany), Bibactyl® (*B. subtilis*, Vietnam), Bio-Kult® (*B. subtilis*, a.o. UK), Biosporin® (*Bacillus subtilis* and *Bacillus licheniformis*, Russia, Ukraine), Calsporin® (*B. subtilis*, Japan), Enterogermina® (*B. clausii*, Italy), and Primal Defense® (*B. subtilis*, a.o., USA).

The promising present and future application of probiotic *Bacillus* sp. seems to be the biocontrol strategy for managing the microflora of the human body and human's closest environment – the modern household. Specific conditions of kitchen and bathroom facilities make them reservoirs of unwanted microbiota biofilms, with *Salmonella* sp., *Listeria* sp., *E. coli* and *Staphylococcus* sp. (Giaouris *et al*., 2015), as well as numerous fungal species like *Exophiala sp., Fusarium sp., Aspergillus sp.* or *Candida* strains (Zupančič *et al*., 2016), algae and protozoa. Hazardous biofilms may have contact with prepared food or be directly transmitted onto the human body, causing a health risk. Cleaning or disinfection products seem to have only minor or transitory effects in long term perspective, as microbial communities gradually develop resistance to antimicrobial agents (Mah & O'Toole, 2001; Myszka & Czaczyk, 2007), while the persistent use of disinfectants may deteriorate human microflora.

In this context, recent evidence for probiotic *Bacillus* sp. ability to block pathogens' signaling system of quorum sensing-managed colonization (Piewngam *et al*., 2018; Pérez-Gutiérrez *et al*., 2013; Noorashikin *et al*., 2016) is of the highest importance. The ability of *Staph-*

ylococcus aureus (*S. aureus*) to increase population density, thus causing the infection, has been successfully inhibited with the key role of *Bacillus* sp. fengycins, a class of lipopeptides, previously known to damage fungus cell membrane (Piewngam *et al*., 2018).

Moreover, the advantage of using spore forming probiotic *Bacillus* sp. is their compatibility with chemical formulations used for household chemistry. The repeatable, regular application of products containing probiotic *Bacillus* species promotes their colonization and proliferation on vulnerable surfaces (Banaszczyk *et al*., 2017), thus assuring microbiological balance. Chemical products containing probiotic species of *Bacillus* sp. may have various physical forms, including paste, atomized spray, liquid under pressure or solution. Examples of household chemicals available on European, African and US markets are presented in Table 6. Microbiological analyses indicate, that widely applied species are *B. subtilis*, *B. licheniformis* and *B. pumilus* (authors' unpublished data), however the information provided by manufacturers is rather scarce, usually omitting exact names of supplemented bacterial species and details concerning whether the formulation contains single or multiple bacterial strains.

Screening for novel, beneficial environmental strains with probiotic qualities within the *Bacillus* genus seems to be a promising future trend to develop new probiotic preparations. New *Bacillus* sp. strains have been recently characterized, showing inhibition against mycotoxigenic fungi aflatoxins (Veras *et al*., 2016) or causing decolonization of methicillin resistant *S. aureus* (*Bacillus* strain TSH58, Chauhan *et al*., 2017).

CONCLUSION

The natural environmental microflora of non-pathogenic *Bacillus* species can become a present remedy for many contemporary issues related to human health and well-being after the civilization lifestyle changes that have dramatically altered our habitat and its microbiological population.

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