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 sp. 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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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^{13}) microbials, 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 RNA 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 (Bjorksten, 1994; Bjorksten 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, 2018):
In 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 broadening 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, respectively focuses the discussion on “live microorganisms bio-products recognized as safe in GRAS Notice Inventory*.

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

<table>
<thead>
<tr>
<th>Bio- products</th>
<th>Name</th>
<th>Source strain</th>
<th>Intended usage</th>
<th>GRAS notification file number</th>
<th>Date of closure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymes</td>
<td>β-glucanase</td>
<td>Bacillus subtilis</td>
<td>Production of beer and potable alcohol</td>
<td>592</td>
<td>2015</td>
</tr>
<tr>
<td></td>
<td>Polygalacturonate lyase (pectate lyase)</td>
<td>Bacillus subtilis</td>
<td>Fruit and vegetable purées and concentrates</td>
<td>114</td>
<td>2003</td>
</tr>
<tr>
<td>Vegetative cells</td>
<td>Bacillus coagulans</td>
<td>Water additive for processing of bananas</td>
<td>559; EC**</td>
<td>2015</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacillus licheniformis</td>
<td>Water additive for processing of bananas</td>
<td>560; EC</td>
<td>2015</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacillus pumilus</td>
<td>Water additive for processing of bananas</td>
<td>561; EC</td>
<td>2015</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacillus subtilis</td>
<td>Water additive for processing of bananas</td>
<td>562; EC</td>
<td>2015</td>
<td></td>
</tr>
<tr>
<td>Inactivated cells</td>
<td>Thermally killed cells</td>
<td>Bacillus coagulans GBI-30, 6086</td>
<td>Liquid and powdered infant formulas/food additive, baked goods, beverages, cereals</td>
<td>670, 725</td>
<td>2017, 2018</td>
</tr>
</tbody>
</table>

*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 compiled 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 (JHCl), 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. helveticus*, *L. plantarum*, *Bifidobacterium lactis*, *B. breve* among others. The LAB species are typically aerotolerant (facultative anaerobic), fermenting, Gram positive, spore formers under the air conditions. The probiotic health benefit of an individual strain depends on the particular characteristics of the host and the actual location of VC (or even a few strains of *Enterococcus* sp.).

The second group of probiotics is referred to as bimodal or allochthonous, 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 BER’s Manual of Determinative Microbiology (Holt et al., 2000), the strains belonging to the *Bacillus* genus are cha-
moorganotrobs, express respiratory or fermentative metabolism, ferment glucose resulting in the production of acid, are positive in the catalase test, and do not reduce nitrate 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-Prączkowiak et al., 2017). Despite being aerobic or aero-tolerant (facultative anaerobic), *Bacillus* sp. still can form endospores 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 Ca²⁺ 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; Ber-
ardeau 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 VC (or even a few strains of *Enterococcus* sp.).

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

<table>
<thead>
<tr>
<th>Genus Bacillus phylogenetic group belonging species</th>
<th>Environmental sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis group</td>
<td></td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td>soil, water, root of tree, seaweed, larva gut, fermented soybean (natto), kimchi</td>
</tr>
<tr>
<td><strong>B. mojavensis</strong></td>
<td>soil of Mojave Desert, soil, river mouth, brackish sediment of the river, spacecraft-associated clean room class ISO 8</td>
</tr>
<tr>
<td><strong>B. vallismortis</strong></td>
<td>desert soil in Death Valley, soil, waste water, river, sand dunes</td>
</tr>
<tr>
<td><strong>B. amyloliquefaciens</strong></td>
<td>fermented soybean (natto), soil, seaweed, animal feces, camel milk, waste water</td>
</tr>
<tr>
<td><strong>B. atrophaeus</strong></td>
<td>soil, air, lake water, decomposed wheat, hay dust, yogurt, fish</td>
</tr>
<tr>
<td><strong>B. licheniformis</strong></td>
<td>fermented bean curd, sediment and water from hot spring, larva gut, human excrement</td>
</tr>
</tbody>
</table>

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
Table 3. Essential features for model probiotic Bacillus species.

<table>
<thead>
<tr>
<th>Feature with range of proposed tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Safety (antibiotic resistance¹, ²; production of toxins¹; genetic stability¹; hemolytic activity¹, ²).</td>
</tr>
<tr>
<td>2) Survivability (survivability in stress conditions e.g. temperature¹; bile concentration²; pH²; sporulating activity¹, anaerobic growth¹).</td>
</tr>
<tr>
<td>3) In vivo compatibility (activity towards invertebrates³; rodents³; mammals³ and human studies ⁴,⁵,⁶).</td>
</tr>
<tr>
<td>4) Colonization ability (cell surface hydrophobicity³; colonization of intestinal mucosa or feces⁴).</td>
</tr>
<tr>
<td>5) Pathogens biocontrol (bacteriocins production⁵,⁶; aggregation with pathogens⁵; barrier function of the intestine⁶, restoring UT, UGT, GIT microfloral⁶).</td>
</tr>
<tr>
<td>6) Immune system interactions (translocation in the gastrointestinal tract (GIT)⁶, anti-inflammatory properties⁶; activating macrophages⁶).</td>
</tr>
<tr>
<td>7) Stimulation of gene/protein markers production (markers of: stress response⁶; adhesion⁶; immune system stimulation⁶; nutrients production⁶; enzymes production⁶).</td>
</tr>
<tr>
<td>8) Specific medical conditions improvement (blood/ heart diseases⁶, anticancer properties⁶).</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Targeted application</th>
<th>Example</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human products: dietary supplements, health foods</td>
<td>Food allergy</td>
<td>Clinical trials on efficiency and safety of association with simethicone in patients with irritable bowel syndrome</td>
</tr>
<tr>
<td></td>
<td>Intestinal and gastrointestinal disorders</td>
<td>Detected ability to affect immunity and inflammatory genes expression in GIT and the reduction of inflammatory diseases of the gut and liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin K production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-cancer properties</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Restoration of microflora responsible for the intestinal and systemic immunity</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal infections</td>
<td>Bacteriotherapy and bacterioprophylaxis, antimicrobial effect of bacteriocins, against broad spectrum of microbes</td>
</tr>
<tr>
<td></td>
<td>Childhood diarrhea</td>
<td>Surface biocontrol components for hospital-dedicated cleaning products</td>
</tr>
<tr>
<td></td>
<td></td>
<td>blocking a pathogen’s signalling system</td>
</tr>
<tr>
<td></td>
<td>Urinary tract infections</td>
<td>Reduction of undesired bacteria (Klebsiella, Proteus, Shigella, Pseudomonas, E. coli) in the urine of elderly patients with slow/static urine flow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Products for topical and oral treatment</td>
</tr>
<tr>
<td>Animal products: feed supplements, functional feed</td>
<td>Pathogens in aquaculture</td>
<td>Veterinary growth promoters and exclusion agents</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal disorders in animals</td>
<td>Biological control agents for aquatic environments (shrimps, shellfish)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activity against bacterial infections (Salmonella enteritidis, pathogenic E. coli) in poultry</td>
</tr>
</tbody>
</table>

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
Thus, the determination of strains and toxins is time-consuming and lack sensitivity or selectivity, and biochemical methods for strains characterizations other enterotoxins. Traditional microbiological plating produce toxins, such as hemolysins, phospholipases, and Bacillus coagulans and Bacillus cereus group, B. mycoides philus mentioned in the Bacillus Ba- tation among healthy adults. The numerous strains of Bacillus sp., type strains with numbers in different microorganism collections and GenBank accession numbers for characteristic sequences.

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

<table>
<thead>
<tr>
<th>Name of the species</th>
<th>Type strain numbers in different collections*</th>
<th>16S rRNA gene sequence accession number (bp)</th>
<th>GenBank accession number (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus amyloliquefaciens</td>
<td>DSM 7, ATCC 23350</td>
<td>AB006920 (274 bp)</td>
<td>FNS97644 (3980199 bp)</td>
</tr>
<tr>
<td>Bacillus atrophaeus</td>
<td>DSM 7264, ATCC49337, NRRL-NRS 2123, NBRC 15539</td>
<td>AB 363731 (1475 bp, partial)</td>
<td>GCA_00159192.5 (4158197 bp, contig)</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>DSM 31, ATCC14579, CCM 2010, LGM66923, NCIB 9373, NCTC 2599</td>
<td>AJ841873.1 (542 bp, partial)</td>
<td>AE016877 (5411809 bp)</td>
</tr>
<tr>
<td>Bacillus coagulans</td>
<td>DSM1, ATCC 7050, NCIB 9365, NCTC 10334</td>
<td>DQ297928 (1549 bp, partial)</td>
<td>ALAS00000000 (3018045 bp)</td>
</tr>
<tr>
<td>Bacillus pumilus</td>
<td>DSM 27, ATCC7061, NCIB 9369, NCTC 10337, CCM 2244</td>
<td>NR_043242 (1434 bp, partial)</td>
<td>ABRX01000001:ABRX01000016 (3833998 bp)</td>
</tr>
<tr>
<td>Bacillus safensis</td>
<td>DSM 19292, ATCC BAA-1126, LGM 26769, NBRC 100820</td>
<td>AF234854 (1434 bp, partial)</td>
<td>ASJD00000000 (373175 bp, shotgun sequence)</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>DSM 10, ATCC 6051, CCM 2216, IAM 12118</td>
<td>LN681568 (1502 bp)</td>
<td>CM000488 (4214598 bp)</td>
</tr>
<tr>
<td>Bacillus toyonensis</td>
<td>BCT-7112T, CECT 876T, NCIMB 14858T</td>
<td>NR_121761 (1546 bp, partial)</td>
<td>CP006863 (4940474 bp)</td>
</tr>
<tr>
<td>Bacillus vallismortis</td>
<td>DSM 11031, NRRL B-14890, BCRC17183</td>
<td>EF433404 (1468 bp)</td>
<td>AFSH01000000:AFSH01000004 (series of shotgun sequences)</td>
</tr>
</tbody>
</table>

*Collection names: ATCC: American Type Culture Collection; BCCM/LMG: Belgian Bacteria Collection; BCRC Bioreource Collection and Research Center (Chinese Taipei); CCM Czech Collection of Microorganisms; CECT Spanish Type Culture Collection; DSMZ: Deutsche Sammlung von Microorganismen und Zellkulturen (eng. German Collection of Microorganisms and Cell Cultures); IAM Culture Collection (Japan); JCM Japan Collection of Microorganisms; NRBC 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 Biosafety level designated as BSL1 (e.g. in American Biological Safety Level 1) for Biological Agents) is assigned to Prokaryotes 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 infec-
tion 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. alcalo-
phillus 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. poly-
myxa, B. thuringiensis and multiple B. cereus strains (ATCC 33018, CA6, CA1, MS1-9, HS23-11) (Gray et al., 2015; Owusu-Kwarteng et al., 2017), using cell cytotoxicity ass-
says, with Ped-2E9-murine hybridoma lymphocytes and CHO-based assays, as well as PCR methods. Hemolysin and lecithinase toxins, emetic toxins, diarrheal toxin, B

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

<table>
<thead>
<tr>
<th>Cosmetics</th>
<th>Body spray, body wash, hand soap, skin cream, skin repair concentrate, toothbrush cleaner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Household chemicals</td>
<td>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</td>
</tr>
</tbody>
</table>
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 adaD2 (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., 2011).

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 insecticidal 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. vulgaris (US origin strains), B. amyloliquefaciens, B. atrophaeus, B. licheniformis or from other closely related clades such as the pumilus group (pumilus, subtilis, safensis), the cereus group (cereus) or alkalophilus 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 et 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 (pON), and long chains of cells with motility factor switched off (pOFF) (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 conservatism (Earl et al., 2012; Jiménez et al., 2016). 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 vulgaris, Bacillus vulgaris 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 & Kazaran, 2012).

An example of a stabilizing solution for Bacillus, but also Lactobacilli and Bifidobacteria, 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; Martin 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; Chavarrí et al., 2012; Martin 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 extraction techniques include alginate solutions (algae derived heteropolysaccharides), whey proteins, pectin, milk or human-like collagen (Chavarrí et al., 2012; Martin 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 (Martin 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 Bacticubity® (B. cereus, France, Germany), Biboacty® (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-Gutierrez et al., 2013; Noorashikin et al., 2016) is of the highest importance. The ability of Staph-
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**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 microbiolog -ical population.

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