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Detection of endospore producing *Bacillus* species from commercial probiotics and their preliminary microbiological characterization

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Abstract

Aim : The main objective of the study was to characterize a mixture of bacterial species, found in commercial probiotic preparation and originally designed for cleaning, biodegradation and wastewater treatment.

Methodology : Lyophilized preparation of environmental strains was microbiologically characterized to determine the growth temperature range, pH resistance and boiling temperature survivability. Gram staining and Wirtz's spores staining were performed for microscopic estimation of cell morphology and sporulation. The MALDI-TOF mass spectrometry method was used to identify bacterial species found in the preparation.

Results : The composition of probiotic bacterial species, isolated from commercial lyophilized preparation, exhibited wide range of growth temperatures, extreme boiling survivability and wide range of pH survivability. The isolated species belonged to *Bacillus* genus, Gram positive and sporulating rods. MALDI-TOF bacterial identification method was carried out for detection of four non-pathogenic, environmental, closely related strains: *Bacillus subtilis, Bacillus mojavensis*, *Bacillus vallismortis* and *Bacillus pumilus.*

Interpretation : The endospore producing environmental *Bacillus* species were detected in commercial probiotics and preliminary characterised. The results of the present study, point out the possible applications of the described *Bacillus* sp. mixture in health, food and cleaning technologies, involving high temperatures or high/low pH processes.

Lyophilized probiotics preparation of environmental *Bacillus* strains

Growth conditions characteristics	Microscopy	MALDI-TOF
⇨ Growth temperature range ⇨ Survivability at various pH ⇨ Boiling survivabilty	⇨ Gram staining ⇨ Wirtz's staining	⇨ Detection of four *Bacillus strains, B. subtilis, B. mojavensis, B. vallismortis and B. pumilus*

Introduction

Probiotics are microorganisms conferring health benefits to both humans or animals, by the mean of pathogenic flora replacement, production of vitamins, immune system stimulation, generation of a chemical/biological environment for gastrointestinal tract functioning (Quigley, 2010; Castelazzi *et al*., 2013; Plaza-Diaz *et al*., 2014). Among them are bacteria naturally residing in the gastro intestinal tract of human, such as *Lactobacillus* sp., and allochthonous or bimodal ones like certain *Bacillus* sp., capable of colonizing the gastrointestinal tract, while being typically environmental microflora. The use of probiotics has become a mainstream application in health, food and cleaning technologies with a number of specialized probiotic-based products.

Studies conducted on animal models have shown positive effects of the bimodal probiotic *Bacillus* sp. These include the protective activity of *Bacillus subtilis* against *Salmonella enteritidis* or pathogenic *Escherichia coli* infection in poultry (La Ragione and Woodward, 2001; La Ragione *et al*., 2001; Thirabunyanon *et al*., 2011). *Bacillus* sp. are also beneficial in establishing rumen microflora in calves, speeding up transition from liquid to solid feed and reducing diarrheal syndrome (Jenny *et al*., 1991). In humans, the benefits of *Bacillus* sp. probiotics include reduction of undesired bacteria (*Klebsiella, Proteus, Shigella, Pseudomonas, Escherichia coli*) in urine of elderly patients with slow/static urine flow (Meroni *et al*., 1983) observed in successful clinical trials. The application of probiotics prevents antibiotic-associated diarrhea in children (La Rosa *et al*., 2003). The use of probiotics in patients with irritable bowel syndrome has been reported effective and safe therapy (Urgesi *et al*., 2013). *Bacillus* sp. probiotics can exercise anti-oxidative stress in humans, including DNA-protective effect (Prazdnova *et al*., 2015). Recent studies have shown the probiotic ability to affect immunity and inflammatory genes expression in gastrointestinal tract and reduction of inflammatory diseases in gut and liver (Plaza-Diaz *et al*., 2014).

Characterization of bacteria in probiotics may enhance knowledge concerning bacterial species content and conditions of the probiotics survival, which is crucial in their applications and implications for assessing potential advantages and drawbacks when used in human- or environment- related industries. Hence present study was undertaken to characterize the bacteria in commercial probiotics BPB-100 and BACILOX® XL 100x.

Materials and Methods

The commercial preparation BPB-100 and BACILOX® XL 100x from Osprey Biotechnics (Sarasota, FL, USA) with the initial CFU values (given by the manufacturer). $\geq 100x10^9$ CFU g^{-1} and $\geq 5.5x10^9$ CFU ml^{-1}, respectively, were used for isolation and characterization of probiotics mixture, namely *Bacillus subtilis*, *Bacillus mojavensis*, *Bacillus vallismortis* and *Bacillus pumilus*. Soy peptone was procured from Scharlau Microbiology (Barcelona, Spain), while other reagents were procured from Sigma-Aldrich (St Louis, MO, USA). Probiotics preparation for subsequent microbiological tests was suspended in LB medium in 1:5 mass ratio, resulting liquid sample for further analyses. Probiotic sample was plated onto solid media: LA, 2YT and TB. Growth temperature range was tested on LA medium and observed after 48 hrs. Survivability at various pH was tested by inoculating the probiotic bacteria liquid sample in 1:100 ratio into the fresh LB medium of pH values ranging from 1-13 and followed by incubation at room temperature for 24 hrs. After incubation, 100 µl of samples were plated on the LA agar and incubated further for 24 hrs at 37°C. The boiling survivability was assayed in LB medium at 100°C, time course samples were taken and streaked on the LA medium for 24 hrs at 37°C incubation.

Microbiological properties were determined using Gram staining and spores Wirtz's staining with malachite green and safranin (Bartholomew and Mittwer, 1952; Hamouda *et al*., 2002). Slide glass preparations were observed under Olympus CX21FS1 light microscope at 1200x magnification.

For the MALDI-TOF mass spectrometry, single colonies on LA were isolated after 24 hrs incubation at 37°C and subjected to MALDI Biotyper (Bruker Daltonics, Billerica, MA, USA) at Laboratoria Medyczne Bruss (Gdynia, Poland). Resulting spectra was compared with intracellular proteins profile databases for microbiological species (Azarko and Wendt, 2011).

Results and Discussion

The commercial probiotics were selected for the study. The detailed microbiology information concerning products are generally not provided by the manufacturers, while probiotics may have substantial impact on the environment and/or human health. The use of probiotics is increasingly popular, thus the presented independent study is useful when planning health, food, cleaning related or environmental usage of probiotics.

Analyses of both Bacilox® probiotics, covering an estimation of the bacterial growth conditions, microscopic examination and MALDI-TOF, showned that preparations contained the same mixture of meso- to thermophilic bacterial species/strains belonging to *Bacillus* genus.

To characterize the probiotics microbiological composition, single colonies from LA medium (3 per each morphological type) were subjected to MALDI-TOF assay. This method determines the identity of the microorganism (Azarko and Wendt, 2011), as microbial protein spectrum is unique for genus/species. The assay allowed to differentiate four closely related *Bacillus* species: *Bacillus subtilis, Bacillus mojavensis, Bacillus vallismortis, Bacillus pumilus,* residing the same lyophilized preparation sample. Results were reinforced by the analysis with standard microbiological assays: Gram staining and

Table 1 : Growth conditions of the bacterial population in the probiotic preparation containing *Bacillus subtilis, Bacillus pumilus, Bacillus vallismortis* and *Bacillus mojavensis*

Growth temperature range	
Temperature (°C)	**Growth intensity**
17	-
18	+
20	++
30	+++
37	+++
45	+++
51	+++
53	+++
55	++
56	+
57	-
Survivability at various pH	
pH	**Growth intensity**
1	+/-
2	+
3	+++
4	+++
5	+++
6	+++
7	+++
8	+++
9	+++
10	+++
11	++
12	+
13	-
Boiling survivability (100ºC)	
Time	**Growth intensity**
0 min	+++
5 min	+++
10 min	+++
20 min	+++
40 min	++
1.5 hr	++
4 hr	+
18 hr	+

Growth intensity rating of probiotic bacteria colonies after 24 hrs incubation at 37ºC on LA agar:- no growth (no colonies); + few bacterial colonies; ++ numerous bacterial single colonies visible; agar partially covered with bacterial film;+++ single coloniesdifficult to distinguish, agar mostlycovered with bacterial film

spores Wirtz's staining (Fig. 1), growth temperature range (Table 1, Fig. 2), pH resistance and boiling temperature resistance (Table 1). All the results corroborated the published characteristics of four *Bacillus* sp. determined. These bacteria are closely related (Roberts *et al.,* 1994; Roberts *et al.,* 1996), *Bacillus vallismortis* and *Bacillus mojavensis* differ from *Bacillus subtilis* in fatty acids composition, whereas being genetically similar in their DNA sequences, proteome conservation and genomes architecture (Earl *et al.,* 2012). High similarity of *Bacillus subtilis* and *Bacillus pumilus* is supported by 16S rRNA gene sequence analysis – out of 5 *Bacillus* groups based on this phylogenetic analysis, *Bacillus pumilus* belongs to the *Bacillus subtilis* group (Berkeley *et al.,* 2008). The four identified strains formed distinct morphological colony variants with *Bacillus vallismortis* and *Bacillus mojavensis* being similar, whereas *Bacillus subtilis* and *Bacillus pumilus* were different in terms of colony morphology, size and growth rate (Fig. 1). The temperature 37ºC was the most differentiating, thus was selected for morphology comparison. Despite morphological differences four strains behaved in a similar manner during microbiological tests, displaying common preferences in growth media type, growth temperature and media pH, survivability at various temperatures. Three tested media, with an increasing supply of nutrition compounds: LA, 2YT and TB, efficiently supported the investigated bacterial growth. LA medium was selected for further use. All further analyses were performed by inoculating media directly with the probiotics preparations to preserve the original species proportions and their possible interactions. Probiotic bacteria temperature growth optimum was determined within temperature range of 16-60ºC for 48 hrs Table 1 shows the temperature dependence of the *Bacillus* sp. probiotic growth. The population grew within the range of 18-56º C, covering facultative psychrophiles or low-mesophilic to thermophilic range. Fig. 2 depicts the temperature-dependant morphological changes in the *Bacillus sp.* bacterial colonies, as at higher temperature the probiotics colonies tended to produce slime. It can be the result of proceeding membrane lipids variation and thermal denaturation of proteins, followed by cell lysis (Ahmed *et al.,* 2008; Munna *et al.,* 2015). Above 50º C, colonies tended to lyse after 48 hrs of prolonged incubation, thus 37-50º C range was optimal temperature for experiments. The ability to survive a long pre-incubation in media with various pH was tested, conditions were mimicked to reflect survivability in the environment, e.g., one relatively rich in trophic compounds, such as human gastro intestinal tract or soil, with pH varying to extreme values. Twenty four hour incubation in LB, pH 1-13 was conducted, followed by plating on LA, at pH 7.0 and 24 hrs incubation at 37º C. Table 1 presents that the examined probiotic *Bacillus* sp. survived prolonged incubation at pH 2-12. The gastrointestinal tract pH of human and mammals below the stomach ranges from 4.5 to 8.5, typically 7-8. These conditions are ideal for probiotic *Bacillus* sp. once they have survived in food fragments the passage through the stomach, with a pH 1-2. Considering the *Bacillus sp.* produce spores, the adaptation to colonize gastrointestinal tract is evident.

To estimate the survivability of *Bacillus* sp. probiotic during high temperature processing, involving extensive boiling, 100ºC resistance was determined. Samples incubated in LB medium at 100ºC were plated onto LA and incubated at 37°C for 24 hrs. Table 1 shows that boiling for 20-40 min resulted in slow decrease in population. On extended to boiling temperature for 18 hrs, probiotic bacteria still survived. This indicates that high temperature food processing does not eliminate probiotic *Bacillus*

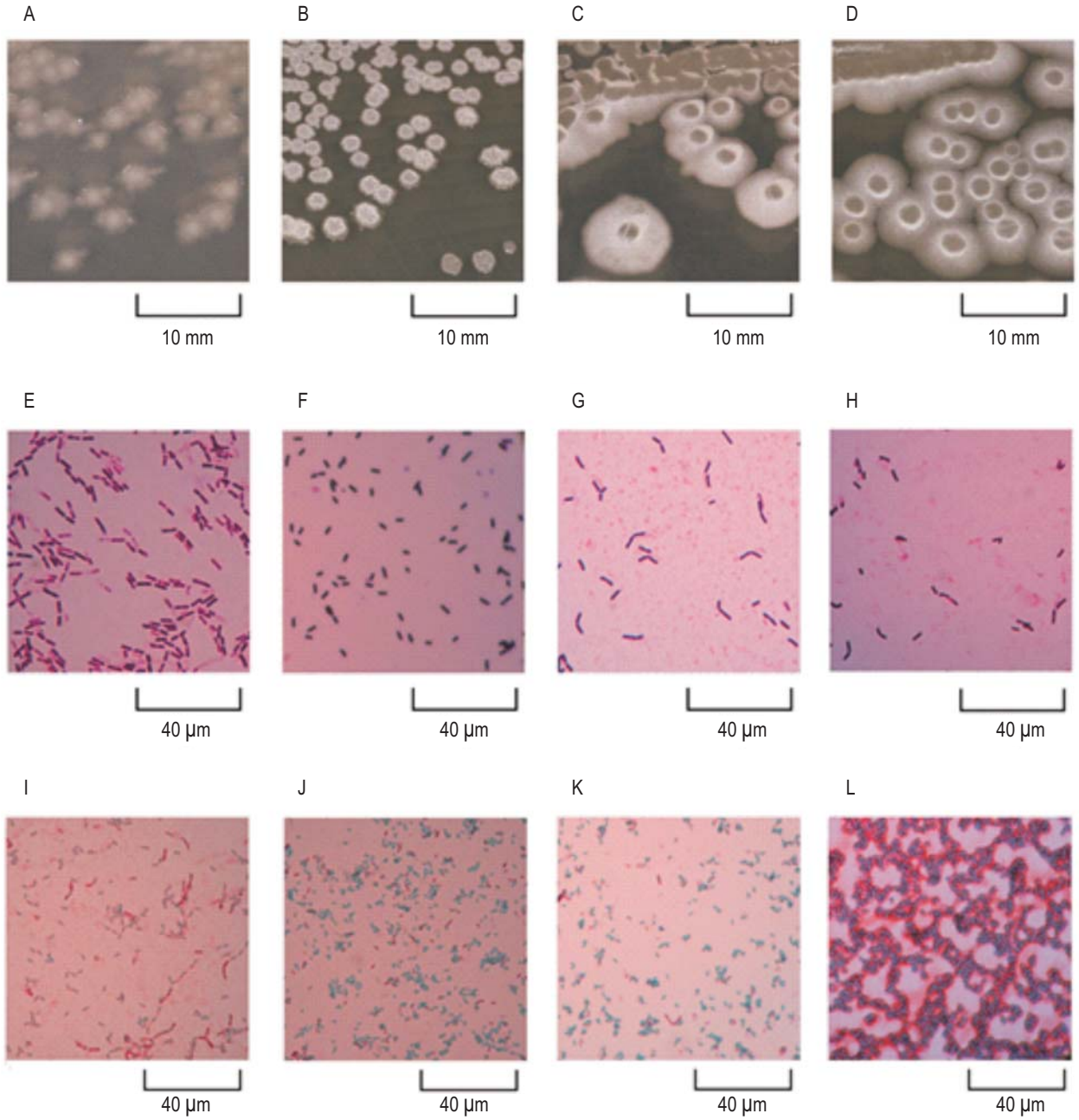

Fig.1 : Bacterial colony and cell morphology of *Bacillus* sp. isolated from the commercial probiotic preparation. *Bacillus* sp. colony growth on LA medium, after 24 hrs of incubation at 37ºC, presenting MALDI-TOF identified species: (A)*Bacillus subtilis*, (B)*Bacillus pumilus*, (C)*Bacillus vallismortis*, (D)*Bacillus mojavensis*. Microscopic examination presenting detected *Bacillus* sp., subjected to Gram staining (E) *Bacillus subtilis*; (F) *Bacillus pumilus*; (G) *Bacillus vallismortis*; (H) *Bacillus mojavensis;* and to Wirtz's spores staining (I) *Bacillus subtilis* spores; (J)*Bacillus pumilus* spores; (K) *Bacillus vallismortis* spores; (L) *Bacillus mojavensis* spores

sp., allowing this beneficial microflora introduction to gastro intestinal tract The survivability of tested probiotic bacteria is an effect of producing spores. Fig. 1 shows green-stained spores within a microscopic preparations after Wirtz's spore staining of examined probiotic *Bacillus* sp.

Wide temperature growth range, survivability of extensive boiling and extreme pH confirm that probiotic *Bacillus* sp. are adapted to the external environment with relatively low temperatures, also to the human gastrointestinal tract, as well as to survive conditions in compost piles, geothermal niches or other

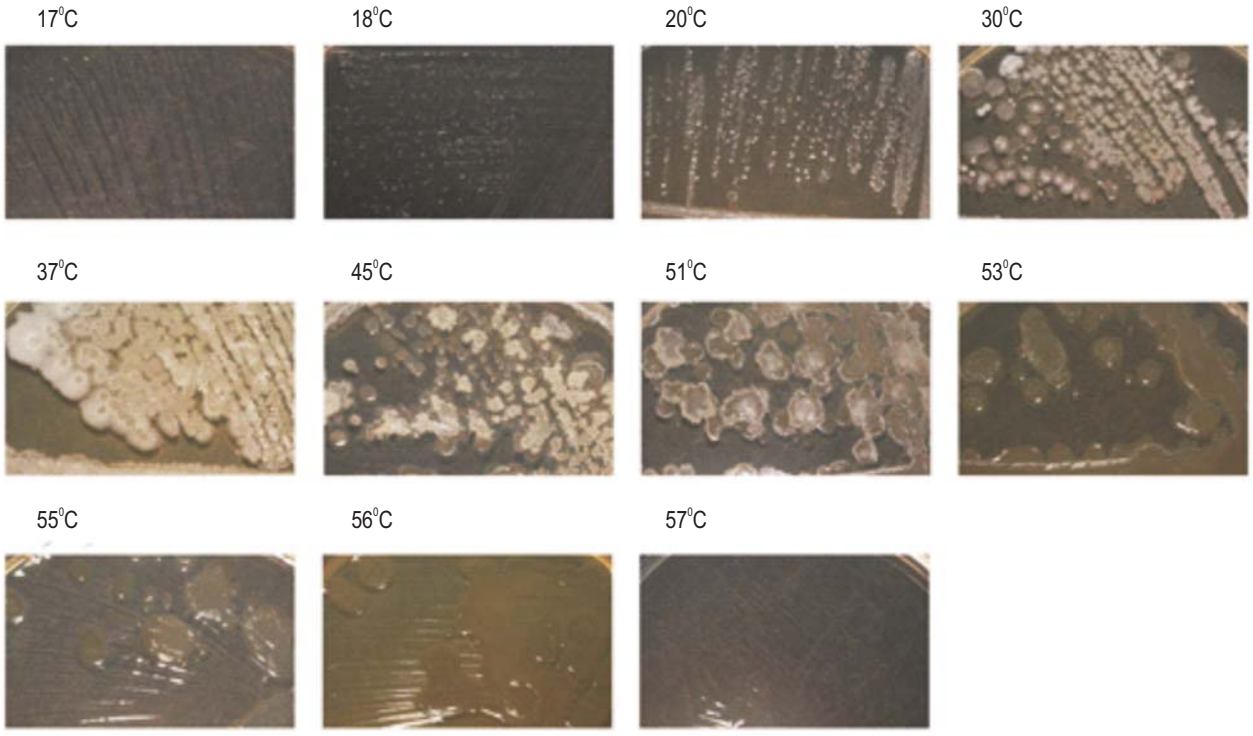

Fig.2 : Morphological changes of the temperature-dependant bacterial colonies of *Bacillus* species from the probiotic preparation. Bacteria grown for 48 hrs on LA medium

harsh conditions. Considering the widespread presence of *Bacillus* sp. in the environment and that detailed cleaning or aseptic food preparation, decreasing or removing the natural microbes has become a practice only in very recent human history, it is certain that *Bacillus* sp. were ingested by humans since ages, being inseparable from human diet. Therefore, aerobically growing *Bacillus* sp. with spores capable of surviving in semi-anaerobic conditions of gastro intestinal tract, should be considered as beneficial commensals of human gastro intestinal tract, not soil microorganisms only. Studies have proved the advantages of *B. subtilis* to survive within the gastro intestinal tract (Hong *et al.,* 2009). Analysing spores concentration of soil and gastrointestinal tract have revealed a high spore number found in human faeces, two orders of magnitude lower than in soil, which is too high, if acquired by food contamination.

The characterised *Bacillus sp.* probiotic mixture has a wide application in biotechnology. The range of *Bacillus subtilis* and *Bacillus pumilus* probiotics usage includes human-related applications, thus being a part of broader therapy e.g., as human dietary supplements (Cutting, 2011) or as surface biocontrol components for hospital-dedicated cleaning products (Vandini *et al.,* 2014). *Bacillus subtilis* probiotics are proposed as biological control agents in the food-producing aquaculture (Verschuere *et al.,* 2001), enhancing the growth by disease-resistance of cultured shrimps or tiger grouper (Cutting, 2011; Yasin, 2016). On the other hand, the composition of *Bacillus subtilis* and its close relatives *B. mojavenisis* and *B. vallismortis,* together with *Bacillus pumilus,* will potentially serve for an environmental remediation procedures, such as wastewater treatment and decomposition of solid organic wastes. These may include high temperature processes or pre-treatments with high/low pH or boiling. *Bacillus* sp. are well known biotechnological sources of extracellularly secreted hydrolase enzymes (Priest, 1977; Barros *et al.,* 2013), mostly heat-resistant or thermostable amylases, lipases or proteases. Currently, *Bacillus subtilis* and *Bacillus pumilus* origin xylanase enzyme (Irfan *et al.,* 2012; Battan *et al.,* 2007,Acharya and Shilpkar, 2016) is being applied for the industrial release of sugars from plants during lignocellulosis biomass pretreatment or in saccharification of food technology wastes

Commercial Bacilox® probiotics preparation consisted of four sporulating species: *Bacillus subtilis, Bacillus pumilus, Bacillus mojavensis* and *Bacillus vallismortis.* The bacteria exhibited growth temperature range of 18-56ºC, boiling and pH extremes resistance. These probiotics are advantageous for bimodal existence in the environment and gastrointestinal tract, having a vast potential in biotechnological applications.

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