

Identification of bacterial species in probiotic consortiums in selected commercial cleaning preparations

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The role of environmentally coexisting microflora that often comprises human commensal microbiome is still underestimated. Modern lifestyle changes include hygienic practices, food preparation and eradication of many contagious diseases. In this context, probiotic microorganisms are biocontrol remedies still under development, solving a number of gastrointestinal and immunological issues, while fighting hazardous microbiological biofilms on different surfaces. Probiotics are mainly associated with Lactic Acid Bacteria, however environmental, non-dairy sources are promising ecological niches of probiotic spore-forming *Bacillus* species. Industrial applications of these “unconventional” probiotics take an advantage of their sporulating activity which greatly enhances their compatibility with chemical formulations used in the household, cosmetic or pharmaceutical chemistry. We have analysed 14 commercially available chemical products, labelled or described to contain a probiotic or biologically active component. It was determined that in the most part they rely on consortiums of spore-forming, very closely related *Bacillus* species, exhibiting bimodal existence in the environment and the gastrointestinal tract (GIT). In addition, we have found a number of non-sporulating species. Overall, the microorganisms found included: *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus pumilus*, *Citrobacter freundii*, *Klebsiella oxytoca*, *Stenotrophomonas maltophilia*, *Serratia liquefaciens*, *Bacillus altitudinis*, *Lactobacillus gastricus*, *Bacillus megaterium*, *Lactobacillus nagelii*, *Aromatoleum buckelii*, *Trichosporon mucoides*, *Clostridium novyi*, *Bacteroides uniformis*. As some of the listed species may become opportunistic pathogens, this raises an important question concerning general safety of probiotics, as apparently the manufacturing procedures do not always lead to microbiologically defined or sufficiently controlled microorganism consortiums.

Key words: *Bacillus* sp. for detergents, *Bacillus* sp. probiotic identification, *Bacillus* sp. probiotic safety, *Bacillus subtilis*, probiotic consortium, probiotic formulations, spore formers

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Abbreviations: B.C., before Christ; BSL, Biosafety Level; CFU, colony forming unit; EC, European Commission; GIT, gastrointestinal tract; GRAS, Generally Recognized as Safe; LAB, lactic acid bacteria; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; OD, optical density; RG, Risk Group; UGT, urogenital tract; UT, urinary tract; w/o, water-in-oil; w/o/w, water-in-oil-in-water

INTRODUCTION

Microorganisms are co-creators of the mankind’s history: the story of struggle with pathogenic microbiota and the ability to take advantage of the species present in the environment. Humans have evolved in a continuous contact with the natural environment. Initially quite unwittingly, we have eventually learned how to subdue microorganisms and benefit from their resources to achieve our own goals. Then, more intentionally with the passing centuries, we gained the ability to perform biotechnology processes. One of the oldest known biotechnological signs of human activity with intuitively applied microorganisms is the ancient Egyptian brewery from Tell el-Farkha (Ciałowicz, 2017). Scientists have confirmed the microbiologically fermented beer to be the major drink in the ancient Egyptian era, dating back to 3700-3500 B.C. in the Lower Egyptian culture Naquada IIB (corresponding to the Early Bronze age). This liquid was preserved for a long time in storage under difficult conditions of ancient times (Ciałowicz, 2017), and was placed as a prayer contribution immediately after bread.

Purposeful application of bacteria may have become a common practice with the growing knowledge of the micro-world. The XVIth century was mind-boggling for society, with Leeuwenhoek’s observations and description (1677) of bacteria seen under a single-lens microscope (Lane, 2015). The origin of contagious, bacterial-derived diseases was explained with germ theory in the late XIXth century when a possibility of transmitting *Bacillus anthrax* between different host organisms had been postulated by Koch in 1877, as well as Pasteur in 1881. Pasteur was the one who also performed the very first intentional and scientifically controlled experiments on fermentation, showing that it can be driven by a bacterial factor (Schmalstieg & Goldman, 2008). In the above context it is particularly worth mentioning the Human Microbiome project, which sees the human body as a supra-organism equally composed of human and microbial components. This international effort has been sustained from 2007 as a global and interdisciplinary endeavour, with investigators publishing their reports in Nature and Public Library of Science, with over 650 publications with over 70000 citations by the end of 2017 (National Institutes of Health; 2018).

The effects of anthropogenic activity cover all environment elements, such as water, soil and air, as well as household environments – including household animals (Schnorr *et al.*, 2016). Microbiome studies are crucial for understanding and solving diseases related to the Western civilisation or the developed countries, with atopic allergy and asthma disorders among others, which in contrast were absent or underrepresented in the ancient

populations. Even today, groups of tribes forming the ancestral and previously uncontacted Indian communities, e.g. in Venezuela (Ravel *et al.*, 2014), present significantly different microbiomes when compared to communities from the Western countries, exposed to modern food, chemicals and the pharmaceutical industry.

Constantly increasing number of commercial chemical products containing probiotics is reaching the market. Probiotic-based formulations dedicated for personal use include cosmetics, e.g. liquid soaps, gels, shampoos, as well as formulas for machine washing, dishwasher and general cleaning products. They are typically present in soaps, detergents, polycarboxylates, non-ionic detergents, phosphonates, enzymes, thickeners, preservatives and dyes.

The probiotic form added to a given chemical formulation is made from a microbiological culture, grown under conditions promoting maximum spore content, subsequently spun down and washed from the remainings of the bacteriological medium. The bacterial cell solution is typically stabilized by emulsion based techniques, involving water-in-oil (w/o) or water-in-oil-in-water (w/o/w) format. These emulsions utilize ionic hydrogels, such as alginates and chitosans, or thermal hydrogels, such as gelatin (gellan gum), xantan, carrageenan, or various types of cellulose polymer derivatives, depending of the final application. A relatively new approach of probiotic preparation form is the use of microencapsulation techniques to stabilize the freeze dried products with sugar or protein cryo-protectants (Martin *et al.*, 2015; Mahidanan *et al.*, 2017).

Our previous findings indicate that the frequently applied species are *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* (Jeżewska-Fraćkowiak *et al.*, 2017). We have presented extensive description of *Bacillus* sp. probiotics intended for the industrial products in a recent review (Jeżewska-Fraćkowiak *et al.*, 2018). The probiotic representatives of *Bacillus* genus are Gram-positive rods, commonly isolated from environmental samples, including water, soil, vegetable and animal origin. Sporulation mechanism (Bernardeau *et al.*, 2017) enables *Bacillus* species to survive under extreme conditions of temperature, water deprivation, osmotic shock, presence of denaturing agents and detergents or radiation. Bimodal probiotic strains of the *Bacillus* genus contribute to the human or animal gastrointestinal (GIT) and/or urinary (UT) and/or urogenital (UGT) tract environment, replacing the unwanted and potentially pathogenic microbiota, thus sustaining the microbiological balance (Hong *et al.*, 2009; Cutting, 2011), after prolonged and rationally planned treatment.

A common characteristic of *Bacillus* species is the ability of producing and often also secreting enzymes, with protease, glucanase, and pectate lyase among others (van Dijk & Hecker, 2013, GRAS Notice Inventory, 2019), as well as antimicrobial agents, with subtilin, coagulin or bacilysin a.o. (Dimkic *et al.*, 2017).

A biotechnological trend for industrial purposes is also creating recombinant strains of “super-secreting cell factories” (van Dijk & Hecker, 2013, GRAS Notice Inventory, 2019). All biologically active compounds impact the common foodborne pathogens found in biofilms in the food industry, as well as in the household locations (Giaouris *et al.*, 2015; Zupančič *et al.*, 2016). Thus, biocontrol activity of *Bacillus* probiotics against *Escherichia coli*, *Salmonella*, *Listeria*, and *Staphylococcus*, along with fungal representatives of *Candida*, *Aspergillus* and others, seems to be the next level remedy in the times of microbiological dysbiosis, resulting

from modern lifestyle changes (Jeżewska-Fraćkowiak *et al.*, 2018).

Commercial use of a microbiological additive in the formula intended for humans is always related to biosafety issues and potential hazard assessments. Different countries, including the European Community members, USA, Canada and others, developed inner systems to evaluate and label bacterial strains according to their safety towards humans or animals. These qualification systems include the Generally Recognized as Safe microorganisms’ inventory (GRAS Notice Inventory, 2019), Biosafety Levels (BSL) or Risk Groups (RG) (Jeżewska-Fraćkowiak *et al.*, 2018).

Bacillus species utilized in industrial preparations must belong to the biosafe and human friendly GRAS strains (GRAS Notice Inventory, 2019), BSL1, RG1. When choosing a potential candidate for a *Bacillus* probiotic additive, one must bear in mind there are also pathogens in this genus, for example *Bacillus anthracis* and certain strains of *Bacillus cereus* (Berkeley *et al.*, 2008; Hwang & Park, 2015).

Human safe probiotic strains, providing a beneficial activity, must be eventually thoroughly characterised (Regulation (EC) No 1924/2006) and labelled, to provide a complete, sufficient and scientifically proven information regarding an actual health impact on humans. In case of industrial chemicals containing a microbiological component this is in fact considered as an Achilles’ heel. Although the European Community developed a system for health claims’ evaluation, it is currently covering probiotics usage in foods only (European Food Safety Authority, 2009).

When a legal framework is concerned, in fact no regulations are available that could define crucial features of a potential probiotic (Jeżewska-Fraćkowiak *et al.*, 2018), while probiotics are mainly mentioned as food supplements or additives. Instead, a strong impact is placed on labelling and standard issues prevalently in foods and supplements.

A detailed list of ingredients, additives, supplements, and improvement agents is an important information an inalienable right of the consumer, regulated in the European Community by a Directive giving the general labelling provisions (Directive 2000/13/EC). While many food products tend to advertise beneficial health effects related to their composition, the regulation prohibits “the use of information that would mislead the purchaser or attribute medicinal properties to food”.

Still, there is an opening for legislator bodies to follow the trends in microorganism usage in different industry branches and keep up with regulations to maintain the customer safety. Moreover, it is the manufacturers’ obligation to exercise their responsibility to release only thoroughly microbiologically characterised products on the market.

Examples of the analysed here industrial specific products, containing sporulating probiotic species include, but are not limited to: all-purpose hygienic cleaner in a tube, solution for direct cleaning use, body spray in an atomizer for hand maintenance, air-conditioning and air biocontrol product in a canister under pressure and a probiotic concentrate for preparing solutions. However, the manufacturer information concerning the microbial species used is usually very limited, incomplete, often omitting precise names of the supplemented bacterial species and details concerning whether the formulation contains a single or multiple bacterial strains, and whether the customer is dealing with a probiotic strain at all (compare the contemporary definition of probiotics, Ha-

venaar & Huis In 't Veld, 1992; Jeżewska-Fraćkowiak *et al.*, 2018). Such products may cause consumer concerns or distrust regarding the unexpected effects of microbial species used, both on the individual's health and the treated items. Thus, the objective of this study was an important human health-related microbiological analysis of several commercial products labelled to contain probiotics. As microorganisms are potent 'biochemical factories' with widely diversified metabolic pathways, producing a variety of secreted enzymes and organic molecules, these findings may contribute to future understanding of biochemistry and molecular biology behind probiotics' action.

As an analytical technique we have used MALDI-TOF, which is a powerful and precise technique based on mass-spectrometry comparison of the protein mass spectrum of the studied organism with those of a reference strains database. For example, it demonstrated its capabilities by differentiating 24 strains belonging to the *Bacillus pumilus* group (Starostin *et al.*, 2015).

MATERIALS AND METHODS

Reagents and equipment. Soy pepton was from Scharlau Microbiology (Barcelona, Spain). All other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). Bacterial cultivation was conducted in sterilised media using an ELMI ESS-207 hot steam sterilizer (Bielsko-Biala, Poland), stabilized-temperature incubators (Binder, Germany), Excela E25 incubator-shaker (NewBrunswick Scientific, USA). Colonies were counted using a CH-20 colony counter (ChemLand, Poland). Bacterial biomass was isolated using a Sigma 1-14K microcentrifuge (SciQuip, UK) and preparative a Sigma 3-18K centrifuge (SciQuip, UK). Bacterial observation was conducted using an MBL 800T light microscope (Olympus, Japan). MALDI-TOF mass spectrometry analysis was performed as described previously, using MALDI Biotyper (Bruker Daltonics, Billerica, MA, USA) at Laboratoria Medyczne Bruss (Gdynia, Poland) (Jeżewska-Fraćkowiak *et al.*, 2017). Probiotic strains' pure cultures were streaked for resulting single colonies on LA and subsequently isolated after 24 hr incubation at 37°C. Spectrum analysis was performed to compare with databases of intracellular protein profiles for microbiological species (Azarko & Wendt, 2011). Quantification and documentation was conducted using a UV custom Canon EOS documentation system.

Bacterial cultivation and observation. Probiotic preparations were suspended and diluted in modified liquid LB media (per litre: soy peptone, 10 g; yeast extract, 5 g; NaCl, 10 g; agar, 15 g) and plated onto three media: modified LA media (soy peptone, 10 g; yeast extract, 5 g; NaCl, 10 g; agar, 15 g), 2YT media (per litre: tryptone, 16 g; yeast extract, 10 g; NaCl, 5 g; for 2YT plates 15g of agar were added) and TB media (per litre: tryptone, 12 g; yeast extract, 24 g; glycerol, 4 ml; KH₂PO₄, 2.31 g; K₂HPO₄, 12.54 g). For the final analysis, modified LB was selected, single colonies were isolated and subjected to MALDI TOF mass spectrometry. Properties, such as media plating, growth temperature profiling, pH resistance, and boiling temperature survivability were determined using standard microbiological methods (Green & Sambrook, 2012). Probiotics' colonies morphology was documented by macro photography under VIS light. Turbidity of commercial preparations was measured after vigorous shaking of relatively viscous prepara-

tion solutions until uniform particle suspension was observed and was then spectrophotometrically measured (OD) using supernatants from spun down preparations as a blank.

Quantitative analysis of vegetative cell number (CFU/ml). To avoid an inhibitory effect of chemicals carried over from commercial preparations on microbial growth, each preparation was shaken for 15 min in an orbital shaker while in the original packaging. Upon complete resuspension of insoluble components (including microorganisms), a 1 ml sample was taken and spun down in a 1.5 ml Eppendorf tube at 5000 × g for 10 minutes at room temperature. The supernatant was discarded and the pellet resuspended in 1 ml PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, Green & Sambrook, 2012). The suspension was spun down as above. The resuspension/washing procedure was repeated 3 times in total. After procedure completion, serial dilutions were made by mixing 0.1 ml of each resuspension with 0.9 ml PBS buffer and dilutions were repeated until 10⁻¹⁰ value was reached, and then placed on ice. Then, a sample of 10 µl from each preparation and dilution was spotted on a pre-dried Petri plate. The plates were incubated at 30°C for 24 h. CFU/ml were calculated as: (1/dilution) × 100 × colony number.

MALDI-TOF bacterial species determination. MALDI TOF mass spectrometry was conducted at Laboratoria Medyczne Bruss, Alab (Gdynia, Poland) and compared to microbiological protein profiles databases. The identification factor values used were as follows: 2.3–3.0 – identification accurate at a species level; 2.0–2.299 – identification accurate at a genus level with possibility of a species accuracy; 1.7–1.999 – identification accurate at a genus level. Whenever several measurements were taken, averaged identification factors are provided.

RESULTS AND DISCUSSION

We have previously analysed a commercial probiotics preparation and determined that it consisted of a consortium of four sporulating species: *Bacillus mojavensis*, *Bacillus vallismortis*, *Bacillus pumilus* and *Bacillus subtilis* (Jeżewska-Fraćkowiak *et al.*, 2017). These bacteria exhibited an extraordinarily wide growth temperature range of 18–56°C, as well as boiling and pH extremes resistance. These probiotics are advantageous for bimodal existence in the environment and GIT. In the current analysis we have found that some of those species are common in 14 preparations analysed here, as well as additional sporulating and non-sporulating bacterial species (Table 1, Fig. 1): *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus pumilus*, *Citrobacter freundii*, *Klebsiella oxytoca*, *Stenotrophomonas maltophilia*, *Serratia liquefaciens*, *Bacillus altitudinis*, *Lactobacillus gastricus*, *Bacillus megaterium*, *Lactobacillus nagelii*, *Aromatoleum buckelii*, *Trichosporon mucoides*, *Clostridium novyi*, and *Bacteroides uniformis*. Below is a short characteristic of bacterial population in each commercial preparation evaluated (Table 1):

Preparation 1: Hand washing liquid with provitamin B5 and with probiotics

The preparation was slightly turbid (OD=0.075) and contained moderate amount of CFUs (1.1×10⁶) assigned to spore-forming *Bacillus licheniformis*, with relatively high MALDI TOF identification factor of

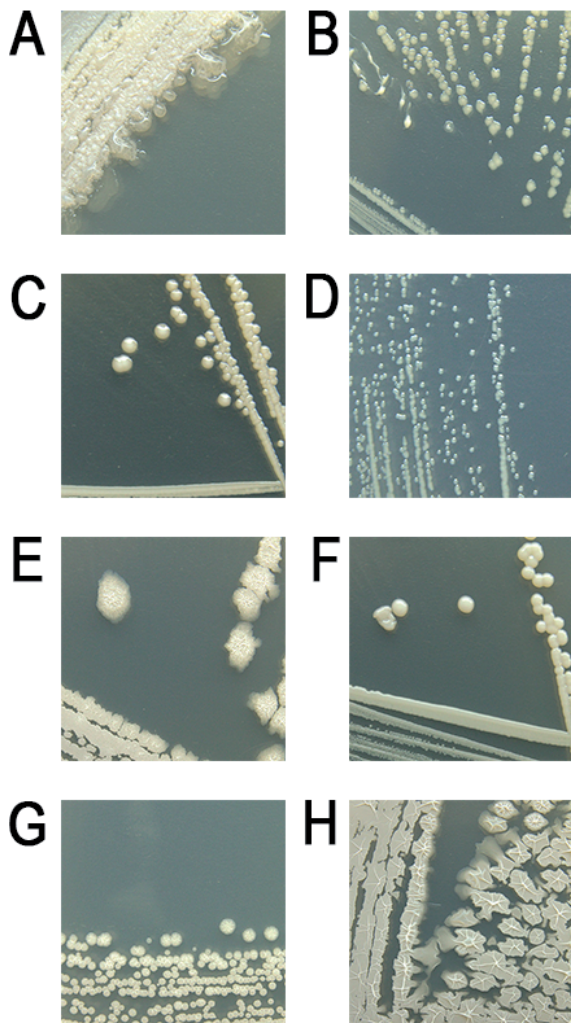


Figure 1. Example of probiotic bacteria colonies detected in the course of commercial preparation analysis.

(A) *Bacillus licheniformis*; (B) *Citobacter freundii*; (C) *Klebsiella oxytoca*; (D) *Stenotrophomonas maltophilia*; (E) *Bacillus subtilis*; (F) *Serratia* sp.; (G) *Bacillus pumilus*; (H) undetermined bacteria.

2.191. The colonies formed two morphological types, however, both were detected by MALDI-TOF as the same species. Thus, the preparation may contain a consortium of 2 very closely related strains of *Bacillus licheniformis*. Alternatively, these may be two forms of the same species with turned on/off motile genes, which is a known phenomenon for *Bacilli* (Kearns & Losick, 2005; van Gestel *et al.*, 2015). These bacteria belong to closely related *Bacillus subtilis* group along with *Bacillus mojavensis*, *Bacillus vallismortis*, *Bacillus amyloliquefaciens* and *Bacillus atropheus* (Wattiau *et al.*, 2001).

Preparation 2: Drain cleaner and septic tank treatment with probiotics

The preparation was highly turbid (OD=0.845) and contained a moderately high amount of CFUs (5.3×10^6) assigned to three non-sporulating species: *Citrobacter freundii* (identification factor 2.184), *Klebsiella oxytoca* (2.111) and *Stenotrophomonas maltophilia* (2.138). Relatively high turbidity as compared to the CFU count suggests that not all cells in the prepara-

tion were viable or a non-bacterial insoluble material is also present in the preparation.

Preparation 3: Baby bottle and dish washing liquid with probiotics

The preparation was of low turbidity (OD=0.158) and contained moderate amount of CFUs (4.5×10^5) assigned to *Bacillus licheniformis* (identification factor 1.944).

Preparation 4: Bathroom cleaner with probiotics

The preparation was of low turbidity (OD=0.097) and contained low amount of CFUs (2×10^4) assigned to *Bacillus subtilis* (identification factor 1.773). This indicates that the bacteria were mostly non-viable in this preparation. This may be due to a more aggressive chemical content of bathroom cleaner formulations, which had eliminated even sporulating bacterial species during prolonged storage. This conclusion would corroborate with the assay repeated over 2 years later, which has shown no CFUs.

Preparation 5: Allergen remover spray with probiotics

The preparation was of low turbidity (OD=0.154) and contained a moderately high amount of CFUs (1.5×10^6) assigned to *Bacillus subtilis* (identification factor 1.906) and *Serratia liquefaciens* (2.228).

Preparation 6: Multi-surface cleaner with probiotics

The preparation was of low turbidity (OD=0.070) and contained a moderately high amount of CFUs (3.5×10^6) assigned to *Bacillus subtilis* (identification factor 1.909). Proportion of turbidity to CFUs count, as compared to other preparations of this manufacturer, indicates that in this preparation the relative proportion of viable cells/spores to non-viable ones is high.

Preparation 7: Foam cleaner with probiotics

The preparation was of high turbidity (OD=0.389) and contained a very high amount of CFUs (2.5×10^8) associated with 3 morphological colony types, assigned to: *Bacillus subtilis* (identification factor 2.012), *Bacillus pumilus* (identification factor 2.159) and a third species/strain, which formed similar colonies to the above listed species, but could not be reliably identified by MALDI TOF.

Preparation 8: Protect gel with probiotics

The preparation was of high turbidity (OD=0.167) and contained a very high amount of CFUs (4×10^7) associated with 3 morphological colony types, assigned to: *Bacillus licheniformis* (identification factor 1.786), *Bacillus pumilus* (identification factor 1.765) and a third species/strain, which formed similar colonies to the above listed species, but could not be reliably identified by MALDI TOF.

Preparation 9: Cleaner with probiotics

The preparation was of extremely low turbidity (OD=0.0015) and contained no CFUs. This suggests that all viable bacteria/spores were lysed by aggressive chemical content.

Preparation 10: Filter spray with probiotics

The preparation was of high turbidity (OD=0.593) and contained a very high amount of CFUs (1×10^7) as-

Table 1. Microbiological characterization of probiotics-containing commercial preparations.

| Preparation No | Commercial preparation | Optical Density (OD) | CFU/ml | Number of strains detected | Identified microbial strains | MALDI-TOF Identification factor |
|----------------|---|----------------------|-------------------|----------------------------|--|---|
| 1 | Hand washing liquid with pro-vitamin B5 with probiotics | 0.075 | 1.1×10^6 | 1 | 1) <i>Bacillus licheniformis</i> | 1) 2.191 |
| 2 | Drain cleaner and septic tank treatment with probiotics | 0.845 | 5.3×10^6 | 3 | 1) <i>Citrobacter freundii</i> 2) <i>Klebsiella oxytoca</i> 3) <i>Stenotrophomonas maltophilia</i> | 1) 2.184 2) 2.111 3) 2.138 |
| 3 | Baby bottle and dish washing liquid with probiotics | 0.158 | 4.5×10^5 | 1 | 1) <i>Bacillus licheniformis</i> | 1) 1.944 |
| 4 | Bathroom cleaner with probiotics | 0.097 | 2×10^4 | 1 | 1) <i>Bacillus subtilis</i> | 1) 1.773 |
| 5 | Allergen remover spray with probiotics | 0.154 | 1.5×10^6 | 2 | 1) <i>Bacillus subtilis</i> 2) <i>Serratia liquefaciens</i> | 1) 1.906 2) 2.228 |
| 6 | Multi-surface cleaner with probiotics | 0.070 | 3.5×10^6 | 1 | 1) <i>Bacillus subtilis</i> | 1) 1.909 |
| 7 | Foam cleaner with probiotics | 0.389 | 2.5×10^8 | 3 | 1) <i>Bacillus subtilis</i> 2) <i>Bacillus pumilus</i> 3) unidentified | 1) 2.012 2) 2.159 3) unidentified |
| 8 | Protect gel with probiotics | 0.167 | 4.0×10^7 | 3 | 1) <i>Bacillus licheniformis</i> 2) <i>Bacillus pumilus</i> 3) unidentified | 1) 1.855, 1.717 2) 1.711, 1.818, 3) unidentified |
| 9 | Cleaner with probiotics | 0.0015 | N.D. | 0 | N.D. | N.D. |
| 10 | Filter spray with probiotics | 0.593 | 10^7 | 2 | 1) <i>Bacillus altitudinis</i> or <i>Bacillus pumilus</i> 2) <i>Bacillus subtilis</i> or <i>Lactobacillus gastricus</i> or <i>Bacillus subtilis</i> (nonreliable identification) | 1) 1.993/1.795 2) 2.060/1.321/1.313 |
| 11 | Biological preparation for the cesspit | 0.001 | 7×10^2 | 2 | 1) <i>Bacillus pumilus</i> or <i>Bacillus subtilis</i> 2) <i>Bacillus megaterium</i> (nonreliable identification) | 1) 1.79/1.581 2) 2.091 |
| 12 | All surface cleaner, concentrate with probiotics | 0.85 | 7×10^2 | 1 | 1) <i>Lactobacillus nagelii</i> | 1) 2.298 |
| 13 | Hand spray cleaner with probiotics | N.M. | 7×10^7 | 4 | 1) <i>Bacillus subtilis</i> or <i>Bacillus licheniformis</i> (nonreliable identification) 2) <i>Aromatoleum buckelii</i> U120 MPB/ <i>Trichosporon mucoides</i> ATCC 204094 THL 3) <i>Bacillus subtilis</i> / <i>Bacillus licheniformis</i> 4) <i>Clostridium novyi</i> A 1025_ NCTC 538 BOG/ <i>Bacteroides uniformis</i> 110706_F9 LUMC | 1) 1.986/1.861 2), 3), 4) no reliable identification |
| 14 | All purpose cleaner with probiotics | N.M. | 10^7 – 10^8 | 4 | 1) <i>Bacillus subtilis</i> or <i>Bacillus mojavensis</i> (nonreliable identification) 2) <i>Bacillus pumilus</i> or <i>Bacillus subtilis</i> (nonreliable identification) 3) <i>Bacillus licheniformis</i> or <i>Bacillus licheniformis</i> 4) <i>Pantoea agglomerans</i> | 1) 1.890/1.832 2) 1.741/1.733 3) 2.123/2.006 4) no reliable identification |

N.D., none detected; N.M., not measured; ID factor scale: 2.3–3.0 reliable identification to the species level; 2.0–2.299 reliable identification to the genus level, probable to the species level; 1.7–1.999 probable identification to the genus level.

sociated with 2 morphological colony types, assigned to: either *Bacillus altitudinis* (identification factor 1.993) or *Bacillus pumilus* (identification factor 1.795), which was identified in a repeated assay from morphologically identical colony taken from the same plate; and either non-sporulating *Lactobacillus gastricus* (identification factor 2.060) or *Bacillus subtilis* (identification factor 1.317), which was identified in a repeated assay from morphologically identical colony taken from the same plate.

Preparation 11: Biological preparation for the cesspit

The preparation was of extremely low turbidity (OD=0.001) and contained a very low amount of CFUs (7×10^2) associated with 2 morphological colony types, assigned to: either *Bacillus pumilus* (identification factor 1.795) or *Bacillus subtilis* (identification factor 1.581), which was identified in a repeated assay from morphologically identical colony taken from the same plate; and *Bacillus megaterium* (identification factor 2.091).

Preparation 12: All surface cleaner, concentrate with probiotics

The preparation was of high turbidity (OD=0.85) but contained a very low amount of CFUs (7×10^2) associated with a single morphological colony type, assigned to non-sporulating *Lactobacillus nagelii* (identification factor 2.298). Proportion of turbidity to CFUs count, as compared to other preparations of this manufacturer, indicates that in this preparation the relative proportion of viable cells/spores to non-viable ones is either low or non-bacterial insoluble material is also present in the preparation.

Preparation 13: Hand spray cleaner with probiotics

The preparation contained high amount of CFUs (7×10^7) assigned to *Bacillus subtilis* (identification factor 1.986) or *Bacillus licheniformis* (1.861). In addition, 3 slightly more morphologically different colony types were detected, which could not be reliably identified by MALDI TOF. Database comparison has indicated possible candidates as: *Aromatoleum buckelii* U120 MPB, *Trichosporon mucoides* ATCC 204094 THL, *Bacillus subtilis*, *Bacillus licheniformis*, *Clostridium novyi* A 1025_NCTC 538 BOG, and *Bacteroides uniformis* 110706_F9 LUMC.

Preparation 14: All purpose cleaner with probiotics

The preparation contained high amount of CFUs (10^7 – 10^8). Four types of colonies were detected, assigned to: *Bacillus subtilis* (identification factor 1.890) or *Bacillus mojavensis* (1.832), *Bacillus pumilus* (1.741) or *Bacillus subtilis* (1.733), *Bacillus licheniformis* (2.065) and bacteria that could not be reliably identified by MALDI TOF. Database comparison has indicated possible candidate as *Pantoea agglomerans*.

The analysis of 14 preparations above indicated that in the most part they rely on consortiums of spore-forming, very closely related *Bacillus* species, exhibiting bimodal existence in the environment and GIT. The results show that even MALDI TOF identification cannot always clearly distinguish between these species (Table 1), as the method relies on identification of produced macromolecule profiles, mostly proteins. Their biosynthesis somewhat varies depending on several factors, such as: growth media used, temperature, and culture growth stage, among others. This indicates that the MALDI TOF method, even though very fast, precise and useful in microbial species determination, should be taken with caution in some cases, such as in case of *Bacillus* species analysis. The results presented in Table 1 indicate that this method still needs refinement and it would be beneficial to complement it with other techniques. However, in general, a reliable identification of *Bacillus* species is a challenge due to their very high genome, proteome, and metabolic similarities, and thus a more specific determination requires DNA analysis techniques targeting unique markers of a given strain, such as the presence of endo- β -1,4-glucanase (Ashe *et al.*, 2014).

Table 1 also includes all potentially identified species, not belonging to the *Bacillus* genus, even with weak species discrimination and showing low ID number after MALDI-TOF evaluation. All of these species should be regarded as inner, unwanted contaminants of bacterial preparations, as they often comprise the biotic or abiotic environmental microflora, including certain cases of human or animal opportunistic pathogenic strains, such as bacteria from the *Enterobacteriaceae* family of facultatively anaerobic Gram-negative rods (*Serratia liquefaciens*, *Kleb-*

siella oxytoca, *Pantoea agglomerans*) or endospore forming, Gram positive *Clostridium novyi*, as well as the *Trichosporon mucoides* fungi (Holt *et al.*, 2000).

Preparation analyses with lower ID values have revealed a potential presence of Lactic Acid Bacteria (LAB) strains, represented by *Lactobacillus gastricus* (Table 1, Preparation 10), that is reported to be first isolated from human milk. These bacteria have the ability to colonize the gastrointestinal tract, show high glycosyl hydrolase activities and they do not produce undesirable compounds (Cardenas *et al.*, 2014). *Lactobacillus nagelii* that was also detected here, was primarily isolated from a wine sample and is known to produce a vast number of various polysaccharide and sugar hydrolytic enzymes (Edwards *et al.*, 2000). As such, it could be helpful in controlling digestive problems due to lack of enzymes in GIT, such as the lactose intolerance.

Concerning non-*Bacillus* strains potentially present in the examined preparations, only *Stenotrophomonas maltophilia* (Table 1, Preparation 2) can be a potential biocontrol agent with biocontrol activity, especially in the agricultural biotechnology. This strain has been widely used until 1980-s as plant growth and germination promoter, reducing pathogenic microflora (Berg & Martinez, 2015). Nevertheless, first reports on human health risk appeared in the beginning of 1980-s, and since then *Stenotrophomonas maltophilia* is regarded as a widespread opportunistic pathogenic strain, with hospitals being the primary site of detection.

Other non-sporulating identified strains include *Citrobacter freundii* (Table 1, Preparation 2). *Citrobacter* bacteria are found in human faeces and urine, as well as in soil, water, sewage and food. They are capable of immobilisation of heavy metal ions and contain enzymes degrading certain carcinogens and produce hydrolytic enzymes, among others (Gill & Schütze, 1999; Wang *et al.*, 2009). Thus, apparently they could be a component of natural microbiota, so inclusion in commercial preparations is justified.

An advantageous use of strains in biotechnological applications may refer to both, *Aromatoleum buckelii* (Ebenau-Jehle *et al.*, 2017), found in soil and water (Table 1, Preparation 13), and to *Bacteroides uniformis* (Table 1, Preparation 13), found in human (including infants) and animal (e.g. pigs) gut and faeces (Grela & Semeniuk, 1999; Benítez-Páez *et al.*, 2017).

A. buckelii belongs to the group of denitrifying microorganisms and is able to degrade aromatic compounds, thus it has been studied for the potential of applications in chemical and pharmaceutical industries, producing environmentally hazardous waste. In turn, *Bacterioides uniformis* seems to have the potential of supporting healthy human/animal organism mechanisms for utilization of diet- or endogenous derived glycans and their intermediates.

All further described strains may pose a potential risk on human health and should be in fact regarded as disadvantageous contaminants of the preparations dedicated for human use.

Serratia liquefaciens strain (Table 1, Preparation 5) is recognized as a potential risk factor for transmitting genes for antibiotic resistance, which are displayed on a conjugative R plasmid, carrying 14 antibiotic resistance genes (Bourouni *et al.*, 2007). Research on gnotobiotic mice, enriched with human gut microflora, has proven the possibility of the R plasmid transfer from *Serratia* cells to *Escherichia coli*.

Opportunistic infections of the human organism, especially through dirty wounds, can be driven by plant as-

sociated *Pantoea agglomerans* (Table 1, Preparation 14) and hospital-related infections may occur among immunocompromised patients (Dutkiewicz *et al.*, 2016). In turn, *Klebsiella oxytoca* (Table 1, Preparation 2), as belonging to the *Klebsiella pneumoniae* type species, is typically residing in water, soil, and vegetables, but evidently poses a risk for pneumonia, bacteremia and other infections (Holt *et al.*, 2000).

Presence of opportunistic pathogens exemplified by *Serratia liquefaciens* raises an important question concerning the general safety of probiotics, as apparently the manufacturing procedures do not always lead to microbiologically defined or sufficiently controlled microorganism consortiums. Their presence in the probiotic preparations also raises a question concerning manufacturing procedures, which in principle should follow detailed regulations (see above).

Besides potential pathogenicity, the presence of some unwanted microorganisms, exemplified by *Serratia liquefaciens* present in the Preparation 5 (Table 1), there is a possibility of antibiotic resistance gene horizontal transfer. Even though the *Serratia liquefaciens* strain detected is not antibiotic resistant, in principle it can easily pick up such resistance from other bacteria via conjugation, transformation or transduction.

Moreover, *Citrobacter freundii*, present in this preparation, can cause urinary tract infections, among other infections (Gill *et al.*, 1999). Nevertheless, since this drain cleaner preparation is devoted to be used outside human body, with adequate application technique, infections can be prevented.

Cellular toxins of different *Clostridium novyi* strains (Table 1, Preparation 13) are known to cause infections in animals (ruminants) and in humans as well. Davies *et al.* reported that the black disease (infectious necrotic hepatitis) and bacillary hemoglobinuria in ruminants is caused by the *C. novyi* type B and C strains (Davies *et al.*, 2017).

Another example of a potentially hazardous microflora is *Trichosporon mucoides* (Table 1, Preparation 13), a fungal strain residing in soil or occasionally comprising the natural microflora of skin or mouth. However, certain groups of patients, especially those immunosuppressed and/or after transplantation, will display *Trichosporon mucoides* related infections of the inner organs, including liver, lungs, gastrointestinal tract and others, which may even result in death (Nettles *et al.*, 2003). Thus, *Trichosporon mucoides* becomes a species of special concern, not only in tropical regions, where it used to be known for causing mild fungal infections of hair.

We have observed very large variation in CFUs count among preparations. Some of them contain such a low amount of bacteria (less than 10^3 /ml), that their usefulness is highly questionable. Whether such low concentration was intended by the manufacturer and implemented during the manufacturing process or arose as a result of the presence of excessively aggressive chemicals in the preparations, resulting in a rapid drop in viable bacteria, is not known. Nevertheless, results presented here point to the fact that supplementing a microbiological component to a chemical product intended for human use still needs formal regulations, concerning human health and the actual claimed results for that probiotic. Moreover, adequate quality control methods should be developed to assess the actual composition of probiotic strains at different stages of the chemical product development.

Conflict of interest

Potential conflict of interest could arise from this study being financed by GRUPA INCO S.A., as this is a competing company. However, the research was conducted at the University of Gdańsk facility with utmost attention to objectivity.

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