# Metabolic diversity of bacteria and yeast from commercial probiotic products illustrated by phenotypic profiling

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**Abstract**

The number of probiotic products in the marketplace is on the rise, gaining momentum along with the upsurge in research on the role of the human gut microbiome in health. Although such products are considered safe for consumption, these probiotic supplements and beverages are not subject to stringent federal regulation for quality. While only certain strains of probiotic microbes have been studied for efficacy in clinical trials, the ingredient labels of commercial probiotics do not always list the strain names. In this study, we investigated the diversity of the bacteria and yeast sold in these products. From a representative selection of commercially available probiotic supplements and beverages, we cultured microbes and identified them with standard methods (16S rRNA gene sequencing, mass spectrometric identification, and Biolog phenotypic profiling), then assessed whether there were strain-specific differences in nutrient metabolism and tolerance to compounds across the isolates from different products. *Bacillus coagulans, Bacillus subtilis, Lactobacillus plantarum, Lactobacillus rhamnosus,* and the yeast *Saccharomyces boulardii* were cultured from 21 commercial probiotic products (fifteen probiotic supplements and six probiotic beverages). Phenotypic profiling revealed metabolic diversity in carbon source usage and tolerance to compounds, within species from different probiotics and from environmental isolates of strains belonging to the same species. Despite this strain level diversity, we observed that up to half of the probiotic supplements for sale in retail and drugstores only list the species, but not the specific strain, on the label. This study highlights that existing labeling conventions for probiotics are insufficient to convey the strain identity and diversity in these products, underscoring the need for clear strain identification and verification of strain-specific probiotic properties, particularly when moving toward therapeutic applications of beneficial microbes.

## Introduction

A widespread awakening in the public and medical community's interest in commensal bacteria for promoting health is currently underway. Accelerated by the ease and affordability of rapid DNA sequencing technology, an avalanche of studies in animal models and humans has linked the microorganisms living on or in the body to countless health conditions. In the decade since the Human Microbiome Project was initiated to understand how microbes impact human physiology and disease [1], hundreds of studies are published each year exploring the roles of the microbiome (the microbial community inhabiting the human body) in a wide range of diseases. The combination of impacts on inflammation, immunity, nutrient metabolism, and even behavior may inextricably tie the healthy balance vs. imbalance (dysbiosis) of the human gut microbiome to one's overall functioning as a healthy human organism. While the composition of bacterial (and fungal, viral, and protozoan) residents of the body is being mapped with increasing phylogenetic detail, many of the organisms are novel and the biological functions of many of their genes remain unknown [2].

This tidal wave of studies on the human microbiome has kindled renewed interest in the idea of adding beneficial bacteria (probiotics) or foods that selectively enhance growth of certain beneficial microbes (prebiotics) to the diet. Supplementing food with microorganisms is by no means new, with fermented foods and beverages common in the diets of cultures around the world, yet mainstream interest and market demand for probiotic foods is growing in parallel with research on the microbiome, nutrition, and health [3]. The promoted advantage of probiotics is the maintenance or restoration of the balance between pathogens and healthy necessary bacteria, via mechanisms such as binding to pathogens, competition for nutrients, antimicrobial production, and modulating the immune system [4]. In addition to their established roles in female reproductive health [5], probiotics have been recognized for myriad effects on digestive health [6].

Obesity has been shown to be correlated with the microbiota, linked to an imbalance in energy homeostasis, and probiotics and/or prebiotics show potential to address this [7]. According to Salazar et al., prebiotic ingestion by obese women caused an increase in *Bifidobacterium* species, attenuated short-chain fatty acid (SCFA) production and thus abated metabolic factors correlated with obesity [8]. Additionally, the consumption of probiotics has been demonstrated to improve insulin resistance syndrome, type 2-diabetes and non-alcoholic fatty liver disease [6]. **ADD_Science paper T2D.** There is evidence that probiotics assist with the production of vitamin B and necessary organic acids and amino acids, with host absorption of vitamins and minerals, and with the production of enzymes such as esterase, lipase and co-enzymes essential to metabolic processes [6]. The composition of the microbiota is also associated with irritable bowel syndrome (IBS), a chronic disorder associated with abdominal pain, distention and abnormal bowel movements, along with low grade inflammation and alterations to the gut immune system [9]. Substantial evidence indicates the efficacy of specific probiotics for alleviating the symptoms of IBS [10]. Other gastrointestinal disorders such as traveler's diarrhea [11] and antibiotic- associated diarrhea [12], have been shown to be treated or prevented with the introduction of probiotics.

Still, the health claims made by many probiotic foods and supplements are often many steps ahead of the science backing the studies. Despite a growing body of clinical trials supporting the specific benefits of well-established strains [10], more carefully designed and controlled studies are needed [13][14]. This gap between the advertised benefits of probiotics and the evidence to support their efficacy, is due in part to the limited regulation on probiotic supplements, which are categorized by the Food and Drug Administration (FDA) as food additives or ingredients rather than drugs. The microbial strains they contain are classified as "GRAS" (generally recognized as safe), but the health claims on packages are not verified by the FDA. With research on beneficial bacteria advancing rapidly, it is expected that if the promise of microbiome-based therapies lives up to projections, then 'precision

84 probiotics' aiming to prevent or treat certain health conditions may soon be classified as drugs and
85 subjected to more stringent regulatory scrutiny and burden of proof for efficacy. Indeed, the end of the
86 2010s may represent the calm before the storm of 'next-generation probiotics' or 'bugs as drugs,'
87 discovered from research on healthy microbiomes, that could be used to treat specific diseases.

88 When probiotics are evaluated for their ability to treat specific conditions in clinical trials, the
89 strains used have demonstrated probiotic properties, yet the strain-level identity of probiotic bacteria is
90 not always provided on the ingredient label [15]. While in some cases probiotic properties are species-
91 or genus-wide [16], this omission raises several concerns, with safety being the foremost. Secondly, for
92 those brands that do not list the strain, it is possible that the included strain does not actually possess the
93 probiotic effects of clinically verified strains. Variability in cultivation and processing during probiotic
94 manufacturing, also raises the possibility that the integrity of the particular probiotic strain is lost over
95 time, through continuous passaging in industrial fermentations. When bacterial populations are cultured
96 repeatedly over time, new stable genetic populations can emerge, such as in ongoing experimental
97 evolution studies of *Escherichia coli* [17] and *Burkholderia cenocepacia* following repeated transfers of
98 the same starting population [18]. It is possible that this could occur after repeated passaging of fast-
99 growing industrial microorganisms, perhaps selecting for better growth under industrial fermentation
100 conditions, and potentially leading to a reduction in their probiotic properties.

101 Identifying microbes at the strain level and ensuring that their beneficial properties are not lost
102 during the culturing and manufacturing process, is imperative for the live organisms to exert their
103 reported effect. The current study aimed to test how well routine genetic and phenotypic methods of
104 microbial identification could distinguish the microbes in common commercial probiotics at the species
105 and strain level, and to investigate the metabolic differences between the bacterial strains in each
106 product. Three approaches were used to identify each probiotic isolate: amplification and sequencing of
107 the 16S rRNA gene, Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass

spectrometry, and the Biolog Microbial Identification system, a 96-well plate multiplex phenotypic assay consisting of 71 carbon source utilization tests and 23 chemical sensitivity tests [19]. We hypothesized that comparing the phenotypic profiles of these common probiotics would reveal differences in their nutrient metabolism (both across different probiotic species, and among strains of the same species). We also explored the prevalence of certain types of bacteria in the marketplace and compared this to the quantity of published research supporting their beneficial effects.

## Methods

### Isolation of bacteria from commercial probiotic products

Probiotic products were purchased in 2016-2017, opened within one month of the date of purchase and bottles were stored at 4 °C. "Single-strain" probiotics contained only one species of live microorganism, and "Multi-strain" probiotics contained two or more species listed on the label; however only one isolated microbe was identified from each product. For those in capsule form, the capsule was aseptically emptied into a microcentrifuge tube containing 1 ml of sterile water and mixed thoroughly. Tablets were ground with a sterilized mortar and pestle and combined with sterile water. Probiotic beverages were sampled using sterile swabs, directly from the original bottle. Using a sterile inoculating loop, the sample was streaked for isolation onto the surface of the appropriate agar growth medium. For culturing *Lactobacillus* species and *Bacillus coagulans*, MRS (de Man, Rogosa, and Sharpe) agar was used, and TSA (Tryptic Soy Agar) was used for *Bacillus subtilis*. Yeast were cultured on SDA (Sabouraud Dextrose Agar) or MRS. Agar plates were incubated at 30-33 °C for 48-72 hours aerobically, and individual isolated colonies were identified. For single strain products, a well-isolated colony was inoculated into liquid medium and incubated for 24 hours, then used to make a frozen glycerol stock by mixing 700 µl of the overnight culture with 300 µl of sterile 50% glycerol (final

131 concentration 15% glycerol) and stored at -80 °C. For multi-strain products, one colony type was
132 selected, and re-streaked for isolation prior to storing as a frozen glycerol stock.

133 To observe cells from isolated colonies, isolated bacteria were Gram stained following the
134 standard procedure [20] and viewed under oil immersion with the 100x objective lens and photographed.
135 As a preliminary differentiation step between *Lactobacillus* and *Bacillus,* endospore staining was
136 performed on 1-week old cultures following the Schaeffer-Fulton procedure (Harley, 2017). Heat fixed
137 smears were covered with a small piece of paper towel, and placed on a rack over a steaming water bath.
138 Malachite green dye (5%) was added on top of paper and heated for 5-7 minutes, adding fresh stain as
139 liquid evaporated. Slides were rinsed in water, counterstained with safranin for 1 minute, then viewed
140 under oil immersion with the 100x objective lens and photographed.
141
142 **Antibiotic susceptibility testing**

143 The Kirby-Bauer test for antibiotic susceptibility was followed with minor modifications. The
144 typical medium for this assay is Mueller Hinton, however Mueller Hinton was used for *Bacillus subtilis*
145 and MRS agar used for *Lactobacillus* spp. Using sterile swabs, overnight liquid cultures were spread in a
146 zig-zag pattern to create a "lawn" of growth on large 150-mm agar plates. A 12-place BD BBL Sensi-
147 Disc Dispenser was used to deposit the following antibiotic susceptibility Sensi-discs (BD, Franklin
148 Lakes, NJ) onto the agar surface: Ampicillin (AM10), Bacitracin (B10), Chloramphenicol (C30),
149 Ciprofloxacin (CIP5), Erythromycin (E15), Gentamicin (GM10), Kanamycin (K30), Neomycin (N30),
150 Penicillin (P10), Streptomycin (S10), Tetracycline (Te30), and Vancomycin (VA30). After 24 hours of
151 incubation at 33 °C, zones of inhibition were measured and compared to the known diameter sizes for
152 susceptibility on the reference Zone Diameter Interpretive Chart, updated by the National Committee for
153 Clinical Laboratory Standards, accessed in (Harley, 2017). Susceptibility to each antibiotic was recorded
154 as susceptible (S), resistant (R), or intermediate (I) based on the diameter of the zone of inhibition.

155

156 **BIOLOG Microbial identification and metabolic profiling**

157 Freshly grown (24-48 hr) colonies, from TSA or MRS agar, were used for microbial
158 identification on GenIII microplates with the BIOLOG semi-automated system (Biolog, Hayward, CA)
159 following the manufacturer's instructions. Bacteria were added to the appropriate inoculating fluid and
160 transmittance (T) was measured and adjusted to 90-98% T. For *Lactobacillus* species, Inoculating Fluid
161 C (IF-C) was used. For *Bacillus* and other species, either Inoculating Fluid A or B (IF-A or IF-B) was
162 used. Conditions are listed in **Table S1.** Suspended cells were dispensed with an automatic multichannel
163 pipettor into the GenIII 96-well microplate (100 µl per well). The GenIII microplates were incubated at
164 33 °C for 16-48 hours and read using the MicroLog™ plate reader and associated software (Biolog,
165 Hayward, CA) once the positive control well A10 turned purple (typically at 20-24 hr of incubation).
166 Positive growth responses are indicated by a color change based on redox dye chemistry. Identification
167 is made by the GENIII MicroStation™ software, which compares the phenotypic fingerprint with a
168 fingerprint database of known bacteria [21]. Similarity (SIM) scores are assigned reflecting how well
169 the isolate matches the pattern in the database, and an identification is given if the SIM score is >0.6.
170 Bacterial identifications and SIM scores were recorded, and the plate image was saved for later analysis.
171 The GenIII Microplate reference layout for each microorganism was also saved (see example in **Figure**
172 **2D**). The results from all Biolog plates were transcribed into a single summary table, using a "P" for
173 positive reaction wells (purple), representing growth and utilization of a carbon source . Wells scored by
174 the software as borderline (light color half-moon in Figure 2D, could be positive or negative) [21] were
175 recorded as "h" for half in the results tables.

176 For yeast, colonies grown for 48-72 hour on SDA were inoculated into 10 ml sterile water, and
177 the cell suspension was adjusted to 50% transmittance, then pipetted into the wells of a YT microplate.

178 YT plates were incubated at 26 °C for 24-72 hours, and analyzed at 24, 48, and 72 hours using the
179 MicroLog™ plate reader until an identification was made.
180
181 **PCR amplification and sequencing of 16S rRNA gene**
182 Polymerase Chain Reaction (PCR) was performed using DNA obtained directly from bacterial
183 colonies. To amplify the near full-length 16S rRNA gene, the primers 27F (5'-
184 AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used. On
185 ice, a Master mix was prepared, containing water, buffer, MgSO4, dNTPs, and primers following the
186 manufacturer's instructions for HotStart KOD Polymerase (Millipore Sigma, St. Louis, MO). Aliquots
187 of 50 µl of the Master mix were added to labeled 8-strip PCR tubes, and a sterile 100 µl pipette tip was
188 used to pick a pinpoint amount of a bacterial colony and transfer the cells directly into the appropriate
189 PCR tube. Amplification reactions were run on a Bio-rad Thermocycler (Bio-rad, Hercules, CA) using
190 the following cycling conditions: denaturation at 95 °C for 2 minutes, 35 cycles of: [95 °C denaturation
191 for 20 seconds, 48 °C annealing for 20 seconds, 70 °C extension for 35 seconds], followed by a final
192 extension step at 70 °C for 3 minutes. After visualizing the 1450-bp amplicons from each PCR reaction
193 using gel electrophoresis, the PCR amplicons were purified using the QIAquick PCR Purification Kit
194 (Qiagen, Valencia, CA). The DNA purified from the PCR reactions was quantified using a Take3 micro-
195 volume plate with the Gen5 Microplate Reader (Biotek Instruments, Winooski, VT). DNA samples
196 were prepared with forward or reverse primer and PCR products were sequenced by Sanger sequencing
197 at the Yale University DNA Analysis Facility on Science Hill, using an Applied Biosystems Genetic
198 Analyzer (New Haven, CT). The .abi files were downloaded and DNA chromatograms were viewed and
199 trimmed using Geneious bioinformatics software (http://www.geneious.com/). Resulting forward and
200 reverse sequences were searched against sequences in the Genbank non-redundant (nr) nucleotide

201 database using Standard Nucleotide BLAST (blastn), and the top-scoring four hits were recorded for
202 each organism.
203
204 **Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry**
205 Bacteria from frozen stock cultures were transferred to MRS or TSA plates and incubated at 30°C
206 for 48 hours prior to identification. Proteins were extracted using either the on-target method or by
207 using an ethanol-formic acid protocol described by Friewald & Sauer (2009). Cells from isolated
208 colonies were directly smeared onto a disposable FlexiMass™ DS target plate using a sterile toothpick.
209 One µl of 25% formic acid was added to the spot and allowed to air dry followed by the addition of 1 µl
210 of the α-Cyano-4-hydroxycinnamic acid (CHCA) matrix solution. The CHCA matrix solution contained
211 50 mg of CHCA dissolved in a 33/33/33 mixture of acetonitrile/ethanol/dH2O containing a final
212 concentration of 3% trifluoroacetic acid. When the on-target method yielded spectra with poor resolution,
213 proteins were extracted prior to spotting using ethanol and formic acid (Friewald & Sauer 2009). Cells
214 from colonies were dissolved in 300 µl of $dH_2O$ and inactivated by adding 900 µl of room temperature
215 absolute ethanol. The cell suspension was centrifuged twice at 10,000 x g for 2 minutes to remove the
216 supernatant. The pellet was air dried at room temperature for 1 minute and dissolved in 10 µl 70%
217 formic acid. Ten µl acetonitrile was added to the formic acid-cells mixture followed by centrifugation at
218 10,000 x g for 2 minutes at room temperature. The resulting supernatant containing extracted proteins
219 was transferred to a separate tube. One µl of the supernatant was spotted onto the target plate and
220 overlaid with 1 µl of the matrix.
221 MALDI-TOF MS was performed on the AXIMA Confidence $iD^{Plus}$ MALDI-TOF Mass
222 Spectrometer (Shimadzu) using Launchpad software version 2.9.1 and the VITEK® MS Plus Spectral
223 Archive and Microbial Identification System $^{TM}$ (SARAMIS) database, V4.12. Samples were analyzed
224 in the positive linear mode with a laser frequency of 50 Hz and within a mass range of 2000-20,000 Da.

The acceleration voltage was 20 kV and extraction delay time 200 ns. Spectra were generated from 500 laser shots and each target plate was calibrated before samples were analyzed using *Escherichia coli* DH5α. Samples were run in duplicate and spectra acquired by Launchpad were processed by SARAMIS. Each spectrum was assigned a confidence level based on a comparison to SuperSpectra in the SARAMIS database. SARAMIS does not assign a taxonomic name if the confidence levels are below 75%.

**Probiotic product label analysis and literature searches**

To estimate the percentage of products listing specific strains on the label, products were evaluated from four marketplaces: a major online retailer, two drugstore chains, and a retail superstore with brick-and-mortar store locations in Shelton, CT. A search for products from the online retailer was conducted between Jan-April 2017 using the keyword "probiotic" and the name of the following probiotic microbes: *Bacillus coagulans, Bacillus subtilis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus rhamnosus, Bifidobacterium,* and *Saccharomyces boulardii.* At least 20 products for each organism were checked, and if the label image contained a specific strain name or number, this was recorded. For brick-and-mortar stores (visited in April 2018), we counted unique products on the shelves in the probiotic section, examined the labels, and recorded the number of products that listed at least one strain ID on the label. In stores in which a high volume of store-brand generics was present, the products were only counted individually if the listed organisms were different from another store-brand product. Note that products found on the shelves at multiple stores were tallied each time in the count for that store; meaning that the list for each store often contained a high degree of overlap (particularly of the most popular name-brand probiotics).

To assess the representation of each type of probiotic bacteria and yeast in the medical literature, we performed literature searches of the Pubmed.gov database (https://www.ncbi.nlm.nih.gov/pubmed/)

in March 2018. Keywords used were the genus and species name of each probiotic (for *B. coagulans*, we also included the former name, *Lactobacillus sporogenes*), AND "human." The filter "Clinical Trial" was selected to count the number of published clinical trials.

## Results

### Isolation and culturing of probiotic microbes

Pure cultures of bacteria and yeast were isolated from 16 different probiotic supplements, six probiotic beverages, and four environmental sources (Chaas fermented beverage, fruit fly gut, kale, and a leaf). Though most probiotic microorganisms are facultative anaerobes, their aerobic growth was comparable to growth with $CO_2$ Gaspaks, and further experiments were conducted under aerobic conditions. *Bifidobacterium* spp. were excluded from this investigation due to their strict anaerobic and fastidious growth. The main bacterial species cultured were *B. coagulans, B. subtilis, L. plantarum,* and *L. rhamnosus,* with typical colony morphology on TSA or MRS agar under aerobic conditions shown in **Figure 1.** While the *Lactobacillus* species have very similar colony appearance (off-white to white, circular, creamy), the two *Bacillus* species are quite distinct, with *B. coagulans* colonies showing irregular edges, translucent tan color, and less robust growth. The colonies of *B. subtilis* have more rapid, spreading growth, and are opaque off-white in color, raised, and wrinkled. Gram staining confirmed that all bacteria were Gram-positive rods. As expected, spore staining revealed that only the *Bacillus* species formed spores (not shown). *B. coagulans* formed visibly stained spores only when the bacteria were grown on TSA, not on MRS. In one single-strain probiotic labeled as "*Bacillus coagulans,*" both *B. coagulans* and *B. subtilis* were cultured repeatedly from the capsule, indicating contamination within the original product.

### Identification of isolated microbes

**Table 1** summarizes the number of identifications, obtained using each of the three methods, that matched the listed species on the label.

**Table 1. Number of correct bacterial species identifications by each method**

| Name | # of Products tested | Correct ID with 16S sequencing | Correct ID with MALDI | Correct ID with BIOLOG |
|---|---|---|---|---|
| *Bacillus coagulans* | 4 | 3 | 2 | 0 |
| *Bacillus subtilis* | 3 | 2 | 3 | 3 |
| *Lactobacillus plantarum* | 5 | 4 | 3 | 5 |
| *Lactobacillus rhamnosus* | 4 | 3 | 4 | 3 |
| Other *Lactobacillus* spp. | 2 | 2 | 0 | 0 |
| Total | 18 | 14 (78%) | 12 (67%) | 11 (61%) |

The species identifications obtained by each method, for each isolate, are listed in **Table 2.** Each isolate was assigned a Code number to de-identify the product brand. Table 2 includes a total of 26 isolated organisms. For the *S. boulardii* probiotic yeast, the only method used was Biolog identification, and two out of three products listing *S. boulardii* were correctly identified with the Biolog system. This total exceeds the 18 total products listed in Table 1, because it includes microbes for which the identity was unknown prior to analysis (some probiotic yeasts, and environmental isolates from the Chaas fermented beverage, fruit fly gut, and leaves). Seven of the products listed the specific strain identification (Strain ID) on the label: *B. coagulans* GBI-30 6086 (patented as GanedenBc$^{30}$®), *B. subtilis* DE111, *L. plantarum* 299V, *L. rhamnosus* GG, and *L. rhamnosus* LCR35 (**Table 2**).

**Table 2. Species identifications of probiotic microbes using three methods**

| **Source** | **Code** | **Label Identification** | **16S sequence BLAST Result** | **MALDI Result** | **Biolog Result** |
|---|---|---|---|---|---|
| ***Bacillus coagulans*** | | | | | |
| Probiotic Beverage | 5 | *Bacillus coagulans GBi-30 6086* | *Bacillus coagulans* | *Bacillus coagulans* | No ID |

| | | | | | |
|---|---|---|---|---|---|
| Probiotic Beverage | 6 | *Bacillus coagulans GBi-30 6086* | *Bacillus coagulans* | *Bacillus subtilis* | *Sporolactobacillus kofuensis* |
| Single-strain Probiotic | 8 | *Bacillus coagulans* | contamination: *Bacillus subtilis*** | *Bacillus coagulans* | *Lactobacillus paracasei* |
| Single-strain Probiotic | 14 | *Bacillus coagulans GBi-30 6086* | *Bacillus coagulans* | *Lactobacillus pentosus/plantarum* | *Brochothrix campestris* |
| ***Bacillus subtilis*** | | | | | |
| Single-strain Probiotic | 9 | *Bacillus subtilis* | *Bacillus* sp. strain BCBT29 | *Bacillus subtilis* | *Bacillus atrophaeus/subtilis* |
| Multi-strain Probiotic | 13 | *Bacillus subtilis* | *Bacillus subtilis* | *Bacillus subtilis* | *Bacillus subtilis* |
| Multi-strain Probiotic | 15 | *Bacillus subtilis* DE111 | *Bacillus subtilis* | *Bacillus subtilis* | *Bacillus subtilis* |
| ENV: leaf surface | 20 | N/A (environmental) | *N/A* | *N/A* | *Bacillus atrophaeus/subtilis* |
| ***Lactobacillus plantarum*** | | | | | |
| Single-strain Probiotic | 2 | *Lactobacillus pentous/plantarum* | *Lactobacillus plantarum* | *Lactobacillus pentosus/plantarum* | *Lactobacillus plantarum* |
| Multi-strain Probiotic | 11 | *Lactobacillus plantarum complex* | *Lactobacillus plantarum* | No ID | *Lactobacillus plantarum* |
| Multi-strain Probiotic | 12 | *Lactobacillus plantarum* | *Lactobacillus plantarum* | *Lactobacillus plantarum* | *Lactobacillus plantarum* |
| Probiotic Beverage | 16 | *Lactobacillus plantarum 299V* | *Lactobacillus plantarum* | *Lactobacillus pentosus/plantarum* | *Lactobacillus plantarum* |
| ENV: Fruit fly gut | 19 | N/A (environmental) | *Lactobacillus plantarum* | *N/A* | *Lactobacillus plantarum* |
| ***Lactobacillus rhamnosus*** | | | | | |
| Single-strain Probiotic | 1 | *Lactobacillus rhamnosus* GG | *Lactobacillus rhamnosus* | *Lactobacillus rhamnosus* | *Weissella viridescens* |
| Multi-strain Probiotic | 7 | *Lactobacillus rhamnosus* | *contamination: *S. epidermidis* | *Lactobacillus rhamnosus* | *Lactobacillus rhamnosus* |
| Single-strain Probiotic | 10 | *Lactobacillus rhamnosus* LCR35 | Lactobacillus rhamnosus | *Lactobacillus rhamnosus* | *Lactobacillus rhamnosus* |
| Multi-strain Probiotic | 17 | *Lactobacillus rhamnosus* | *Lactobacillus rhamnosus* | *Lactobacillus rhamnosus* | *Lactobacillus rhamnosus* |
| ENV: fermented milk beverage "Chaas" | 18 | N/A (environmental) | *Lactobacillus casei* | *Lactobacillus sp.* | *Lactobacillus rhamnosus* |
| **Other *Lactobacillus spp.*** | | | | | |
| Single-strain Probiotic | 3 | *Lactobacillus gasseri* | *Lactobacillus gasseri* | No ID | *Streptococcus oralis or Paenibacillus sanguinis* |

| | | | | | |
|---|---|---|---|---|---|
| Single-strain Probiotic | 4 | *Lactobacillus acidophilus* | *Lactobacillus acidophilus* | No ID | No ID |
| **YEAST** | | | | | |
| Kombucha 1 | 21 | *Saccharomyces boulardii* | *N/A* | *N/A* | *Saccharomyces cerevisiae a/Tor.pretorein* |
| Kombucha 2 | 22 | *Saccharomyces boulardii* | *N/A* | *N/A* | *Pichia chmeri A* |
| Kombucha 3 | 23 | N/A | *N/A* | *N/A* | *Hanseniospora guillermondii/uvarum* |
| Probiotic rice wine | 24 | *Saccharomyces cerevisiae* | *N/A* | *N/A* | *Saccharomyces boulardii* |
| Multi-strain probiotic | 25 | *Saccharomyces boulardii* | *N/A* | *N/A* | *Saccharomyces boulardii* |
| ENV: Kale leaf | 26 | N/A (environmental) | *N/A* | *N/A* | *Sporidiobolus pararoseus A* |

295

296

297 **Identification by 16S rRNA gene sequencing**

298 Molecular identification by sequencing the 16S rRNA gene is currently a routine method of
299 confirming species identity. The universal primers 27F and 1492R were used to amplify the near full
300 length 16S gene, using direct colony PCR. Since this gene sequence is generally not variable enough to
301 distinguish between strains of the same species, we used 16S rRNA sequencing primarily as a quality
302 control check to ensure that we had indeed isolated the correct species from each product. For those
303 products listing the Strain ID on the label, the top-scoring BLAST hit of the 16S sequence usually did
304 not match the exact strain name (with the exception of *L. rhamnosus* strain "GG."), and the % sequence
305 identity of the five top-scoring BLAST hits were typically identical (**Table S2).** In most cases, the
306 nucleotide BLAST search of the forward and the reverse sequences yielded different strain names of the
307 expected species (**Table S2**). Several of the incorrect IDs (strain codes 7 and 8) may be attributed to
308 contamination during the procedure (Table 2).

309

310 **MALDI-TOF identification**

Using Matrix Assisted Laser Desorption Ionization Time of Flight Technology Mass Spectroscopy (MALDI-TOF), the mass fingerprints of 15 bacteria isolated from commercial probiotics or environmental sources were obtained and identified at the genus and species level. For 12 out of 18 isolated strains the MALDI identification matched that of the probiotic label (**Tables 1+2)**. The "Chaas" isolate was a homemade fermentation so the bacterial identity was not labelled; MALDI- TOF MS identified this strain only on the genus level (*Lactobacillus sp.*). All *Bacillus subtilis* isolates were correctly identified by MALDI-TOF MS. *Bacillus coagulans* strains were not as consistently identified by this technique. The probiotic drink isolate (strain 14) was identified as *Lactobacillus pentous/plantarum*, while the probiotic label indicated the strain was *Bacillus coagulans*. *Bacillus coagulans* (strain 6) was misidentified by MALDI as *B. subtilis.* Several *Lactobacillus* strains were unable to be identified: *L. acidophilus, L. gasseri,* and one *L. plantarum* (strain 11.) However, other *L. plantarum* isolates were successfully identified so this species is represented in the SARAMIS database. If a given peptide mass spectrum matched the database with a confidence score <75%, no identification in SARAMIS could be reported; confidence interval scores are provided in **Table S3**.

**Biolog Identification & Phenotypic Profiling**

Of the 18 probiotic bacterial isolates, 11 were correctly identified using the Biolog Microbial ID system (**Table 1, Table 2).** For 17 of these strains, the expected identity of the bacteria was given on the product label, however the strain isolated from the "Chaas" fermented milk beverage was not known in advance. Among the *Lactobacillus* isolates, *Lactobacillus plantarum* strains were the most amenable to identification with the Biolog system. All of the *L. plantarum* strains isolated from probiotic products were correctly identified, with SIM scores >0.6 (**Table S1**). One *L. rhamnosus* strain yielded an incorrect identification, erroneously reading as *Weissella viridescens.* Neither *L. acidophilus* nor *L. gasseri* were identified correctly with the Biolog assay. For the *Bacillus* strains isolated from probiotics,

335 *Bacillus subtilis* was more readily identified using the Biolog system. Three *B. subtilis* isolates from
336 different products were accurately identified, while none of the three *B. coagulans* isolates came up as
337 *B. coagulans*. Factors such as poor growth, use of non-optimal inoculating fluid or medium, or
338 inappropriate incubation conditions may have had adverse effects on the phenotypic expression pattern.
339 The time of incubation of the GenIII microplate is critical to a correct identification, as is the growth
340 medium. While the Biolog manufacturer recommends BUG-B agar, we observed better growth of *B.*
341 *coagulans* on MRS medium, so MRS was used.

342 **Figure 2** summarizes the results from the Biolog phenotypic profiling for each strain, with
343 carbon source utilization patterns shown in Figure 2A and tolerance to environmental stressors (acidity,
344 salt, and various compounds) in Figure 2B. The reference pattern stored in the Biolog GenIII database is
345 displayed in the top row for each species. The darker color "P" wells indicate a strong positive, while the
346 lighter "h" wells indicate borderline results (Figure 2D); the preferred carbon sources are typically used
347 up more rapidly and completely, yielding a dark purple well, while less preferred substrates may be used
348 more slowly and incompletely[21]. Results of antibiotic susceptibility testing for 12 antibiotics using the
349 disc diffusion (Kirby-Bauer) method are also included in Figure 2. Strain-specific differences were
350 observed for all of the species, in both carbon source utilization and environmental stress tolerance.

351 The Biolog system is also capable of identifying fungi such as yeast, using specific YT yeast
352 plates and the YT database. After optimizing the time and temperature (72 hours at 26 °C), we identified
353 yeast strains isolated from four probiotic drinks, one probiotic supplement and the surface of a kale leaf
354 with Biolog YT (**Table 2, Figure 2C).** Three of these (strain codes 21,22, 25) were labelled as
355 containing *Saccharomyces boulardii*, and the correct identification was obtained for two out of three.
356 The metabolic utilization patterns of 35 carbon sources were compared for these probiotic yeasts and
357 can be viewed in **Figure 2C.** The sugars glucose (Well D6/D7), turanose (Well A8), and maltose (A3)
358 had clear positive growth in the all *S. boulardii* isolates. Inulin (Well A12), a common prebiotic fiber,

was utilized by several strains, although the reference pattern for *S. boulardii* used in the Biolog database is negative for inulin (Figure 2C).

**Evaluation of product labels and quantification of published clinical studies**

One objective of this project was to estimate the percentage of probiotic products currently on the market that list the specific strain ID of the bacterial or yeast species on the ingredient label. Products available from a major online retailer, at two drugstore chains, and at a retail superstore were evaluated by reading the ingredient label and recording whether or not each product listed an alphanumeric strain ID (often the patented name of the strain). **Table 3** summarizes these product counts from four retail sources. With this approximation we saw that an average of 49% (ranging from 34-69%) of products contained specific strain information on the label.

**Table 3. Strain identifications listed on probiotic product labels at retail stores**

| Store | # products checked | # products with Strain ID | % with Strain ID | % without Strain ID |
|---|---|---|---|---|
| Major online retailer | 121 | 41 | 34% | 66% |
| Drugstore 1 | 28 | 13 | 46% | 54% |
| Drugstore 2 | 21 | 10 | 48% | 52% |
| Retail superstore | 26 | 18 | 69% | 31% |

To examine the number of clinical studies performed on these probiotic strains, providing context for the amount of evidence supporting their health claims, we searched the biomedical literature database Pubmed.gov, for clinical trial publications on each species. **Figure 3** shows the total counts of published clinical trials for eight common probiotic microbes. The well-established probiotic species, *Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Bifidobacterium infantis* (a substrain of *B. longum)* were included in this literature search despite not being characterized in the current study. As shown in **Figure 3,** *L. acidophilus* and *L. rhamnosus* have each been studied in over 300 clinical trials to

date. In contrast, there are fewer clinical trials published on *Bacillus* probiotics (49 found for *B. coagulans* and 47 for *B. subtilis*). Evaluation of the content or quality of these studies was not performed.

## Discussion

We isolated and cultured microbes from commercially available probiotic supplements and beverages and used three approaches to identify the bacteria from these products. We then looked for evidence of strain-level diversity between the isolates using phenotypic profiling. Sequencing of the 16S gene and MALDI-TOF mass spectrometry both yielded accurate identifications for a higher percentage of bacteria than the Biolog assay. Sequencing of the 16S rRNA gene has become a standard molecular identification technique since its introduction in the 1980s [22], but is generally not sufficient for strain typing. MALDI-TOF mass spectrometry is a newer method for rapid identification of bacteria, however it requires that the peptide mass spectra already exist in the database [23]. When comparing 16S sequencing, Biolog identification, and other methods for identifying *Lactobacillus* species, only MALDI mass spectrometry was noted for the ability to identify species at the subspecies, or strain level [24]. A study on 148 strains of *Lactobacillus* species isolated from food found that the MALDI technique generated accurate species identifications more often than 16S PCR (93% accuracy vs. 77% for PCR), and the authors suggest that MALDI should be used in combination with genotypic methods for improved reliability [25]. Sato and colleagues (2017) used MALDI-TOF and repetitive sequence based PCR (rep-PCR) for rapid strain typing of strains of *B. coagulans* [26]. This group found a strong correlation between these two methods to successfully distinguish between closely related strains, and reported that carbohydrate utilization patterns correlated well with the MALDI and rep-PCR results for some phylogenetic clusters [26]. We observed agreement in the results from the three methods, with minor exceptions. For strain 1, MALDI and 16S results were in agreement, correctly identifying *L.*

*rhamnosus* (**Table 2**), but the Biolog identification of this isolate was *Weissella viridescens*, a lactic acid bacterium (formerly classified as a *Lactobacillus*), often found in fermented foods [27]. Its pattern of carbon source usage is quite similar to the other *L. rhamnosus* strains. For the "Chaas" isolate, 16S sequencing identified this as *L. casei*, while Biolog suggested *L. rhamnosus.* These species are closely related and belong to the *L. casei* group along with *L. paracasei*.

Several other molecular approaches have been used for distinguishing bacteria at the strain level, such as multi-locus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), or amplified fragment length polymorphism (AFLP). MLST used to be a "gold standard" for differentiating between strains, however this is changing as costs of whole-genome sequencing (WGS) have decreased [28]. PFGE and AFLP have been used to differentiate among probiotic strains of *L. rhamnosus* [29] and *L.plantarum* isolated from various sources [30]. Ceapa and colleagues (2015) identified genotypic clusters of *L. rhamnosus* with AFLP that correlated with functional metabolic clusters determined by Biolog profiling [31]. Due to decreasing costs and improved efficiency, WGS is likely to be the future of microbial strain typing [32]. Phylogenomics approaches have recently been proposed to improve the classification of the diverse *Lactobacillus* genus [33,34], and metagenomic strategies with bioinformatics analyses are in being developed to characterize microbial diversity at the subspecies level in human microbiome communities [35] [36].

Several studies have used comparative genomics to investigate the genetic basis of the probiotic properties and predicted metabolic capabilities of these organisms. Khatri and colleagues compared genomes of *Bacillus coagulans* to *Bacillus subtilis,* and found considerable genetic heterogeneity in *B. coagulans* strains [37], which has been recapitulated in carbohydrate utilization assays [26]. Analyzing the genome of the commercialized *B. coagulans* strain HM-08 uncovered a xylose utilization gene cluster, lending insight to future biotechnological applications for lactic acid production by this strain [38]. Genome analysis has revolutionized the classification and characterization of lactic acid bacteria,

and functional genomics investigation has led to the discovery of novel processes of communication with host cells, that may serve as models for understanding other host-microbe dynamics [39]. Comparative genomics of *Bifidobacterium* species has unveiled substantial diversity carbohydrate metabolism [40] and helped identify the cell surface proteins and exopolysaccharides used to colonize the host intestine [41]. The wide range of polysaccharides used by *B. longum* species has been proposed to aid in their success as early colonizers of the infant gut [42]. By comparing the genome of *Lactobacillus rhamnosus* GG to a less-adherent starter culture strain, a unique genomic island encoding secreted pilins that bind to human intestinal mucus was discovered [43]. These mucus-binding pilins were shown to specifically outcompete vancomycin-resistant *Enterococcus faecium* (VRE) due to homology in the binding site [44].

Despite its lower performance to correctly identify probiotic bacteria, the Biolog 96-well plate assay is a valuable tool for rapidly collecting phenotypic data on 71 carbon sources and 23 environmental stressors in one multiplex assay, and illustrates a critical link between prebiotics and probiotics. By comparing the carbon source utilization patterns of the *Lactobacillus* and *Bacillus* species, we can gain insight on shared and unique metabolic properties of these probiotic strains. The most striking difference between the two *Lactobacillus* species compared to the *Bacillus* species, is their overwhelming preference for sugars. *Lactobacillus plantarum* strains showed a very consistent pattern of sugar utilization, with 14 sugar wells having a positive reaction for all five of the strains tested. These results correlate with comparative functional genomics and metabolic profiling studies on *L. plantarum* [30][45]. *L. plantarum* probiotic strains, but not *L. rhamnosus*, utilized the complex polysaccharide, pectin (Well F1), found in the skins of many fruits. This may be related to its prevalence in plant-associated habitats. Pectinolytic enzymes have been characterized in *L. plantarum* [46] and pectin affects the probiotic phenotype of this species *in vitro* [47]. Likewise, *L. plantarum* grew on gentobiose (Well A6), a rare disaccharide found in the gentian family of plants. Only *L. rhamsosus* utilized the

sugar rhamnose (Well C8). The antibiotic susceptibility profiles of *L. rhamnosus* vs. *L. plantarum* are slightly different (**Figure 2)**. Like most lactobacilli, they are naturally resistant to vancomycin (Well F10) due to absence of D-ala in the peptide crossbridge of their cell walls [48], but *L. plantarum* strains also displayed resistance to ciprofloxacin. The three *L. rhamnosus* strains tested were susceptible to penicillin.

As a soil microbe subject to nutrient limitation in the environment, *B. subtilis* is known to be much more versatile in its metabolism [49], and this is reflected in its Biolog metabolic profiles. A variety of amino acids are utilized, such as L-alanine (Well E3) and L-aspartic acid (Well E5) (**Figure 2A**). These wells turned purple (positive) more rapidly than some of the sugar wells. *B. subtilis* is the most salt-tolerant of the species investigated, growing at up to 8% NaCl (Well B12), but is more susceptible to antibiotics than the lactobacilli (**Figure 2B).** *Bacillus coagulans*, formerly classified as *Lactobacillus sporogenes* and isolated from spoiled milk in 1915, inhabits ecological niches more similar to lactic acid bacteria than other *Bacillus* spp. [50]. Although the Biolog approach did not correctly identify the *B. coagulans* isolates, their pattern of carbon metabolism is primarily metabolizing sugars, similar to the *Lactobacillus* species, and with the ability to withstand pH 5 (Well A12, **Figure 2B).** While *B. coagulans* is categorized as a "GRAS" ingredient, safety considerations are critical for consumption of *Bacillus subtilis* [15]. It is unclear what the source of *B. subtilis* lacking Strain IDs are, as the Biolog ID of strain 9 (from a single strain probiotic also containing plant extracts) resulted in the same identification, *Bacillus atrophaeus/subtilis,* as the ID for a *B. subtilis* environmental isolate, which we cultured from the surface of a leaf. The profile of this environmental *B. subtilis* was quite similar to the strains from probiotic supplements, with the exception of utilization of quinic acid (well F8), a compound found in plant sources. The spore-forming ability of *Bacillus* species makes them highly stable probiotics that can be easily added to food or gummy supplements, however *B. subtilis* especially merits increased regulation for safety and efficacy.

476 It is well known that strain-level differences occur in the probiotic properties of microorganisms
477 [51]. From survival in the GI tract (by tolerance to acidic pH and bile salts), to adhesive capacity to
478 intestinal cells, to competition with pathogens and production of bioactive compounds, the capacity and
479 efficiency to perform these functions is often strain-dependent [52][53]. Here we show strain-level
480 differences in several environmental stressors, such as salt tolerance (**Figure 2B,** wells B10-B12) among
481 the *Lactobacillus* strains, and considerable variation in nutritional phenotypes, based on profiling of
482 carbon source usage. There is evidence that these two phenomena are related: the food sources and
483 molecular cues that microbes encounter in their environment affect their expression of proteins and
484 compounds (or community-level behavior such as aggregation and biofilm formation) that convey the
485 probiotic's beneficial effect. Further experiments are needed to measure a correlation between the
486 metabolic profiles and probiotic properties of these particular strains, similar to a study of two lactic acid
487 bacteria from the commercial culture FloraMax®-B11 [54]. Several examples of the relationship
488 between nutrient sources and bacterial probiotic phenotype include: increased resistance of *L. plantarum*
489 to gastric juices when growth with pectin or inulin compared to glucose [47]; differences in cell surface
490 hydrophobicity, cell surface protein and exopolysaccharide production of *L. rhamnosus* grown on
491 fructose, mannose, or rhamnose [55]; and increased the adhesion of *Lactobacillus acidophilus* to mucin
492 or intestinal cells in the presence of fructooligosaccharides (FOS), cellobiose, or polydextrose [56]. The
493 prebiotic cellobiose was shown to change surface layer proteins and increase auto-aggregation in two
494 *Lactobacillus* strains [57]**.**

495 A thorough understanding of the nutritional preferences of the commensal bacteria for current
496 and next-gen probiotics will be vital for their translation into effective products, and metabolic profiling
497 can help inform the design of "synbiotic" foods (containing probiotics and prebiotics) [58][6] and
498 "biofunctional" foods (in which microorganisms cause the desired biological or physiological effect)
499 [51]. If end-products of microbial metabolism contribute to the health-promoting effect, it will be

imperative that the target microbes have an ample supply of and access to the carbon sources and environmental signals that lead to synthesis of those end-products. For example, plant glucosides from fruit are metabolized by *L. acidophilus*, which then secrete aglycones that exert beneficial effects on the host [59]. Among the wide array of bioactive compounds produced by lactic acid bacteria are B vitamins, gamma-aminobutyric acid (GABA), bioactive peptides, bacteriocins, and other complex molecules such as exopolysaccharides [51]. Several other recent review articles summarize the health benefits provided by microorganisms in functional foods [60] and explore the idea of how dietary composition can reshape a healthy microbiome to restore functions lost through the Western diet and lifestyle [61][62][7]. In a randomized clinical trial evaluating dietary interventions for type 2 diabetes mellitus, a high fiber diet altered the composition of the gut microbiota, and greater diversity of carbohydrates was associated with improved clinical outcomes [63]. The authors noted strain-specific effects on which active SFCA-producing bacteria were positive responders to the fiber, such as certain strains of *Faecalibacterium prausnitzii* [63].

While traditional probiotic bacteria (lactobacilli and bifidobacteria) have been the subject of research for decades, less is known about the preferred nutritional requirements of other dominant members of the human gut microbiome. Using defined media, Tramontano and colleagues tested the carbon source utilization of gut commensals (including mucin, carbohydrates, and the inhibition of growth of some by amino acids or SCFAs [64]. 'Culturomics' studies combining MALDI-TOF identification with >200 culture conditions optimized for fastidious growth have led to identification of 341 species of bacteria cultured from stool samples and helped optimize culture conditions for these bacteria [65]. Better models to study the gut ecosystem are needed, to understand the context of the metabolic interactions between gut commensals and probiotics.

In conclusion, there is currently a disparity between the composition of marketed probiotics available to consumers and the science backing their claims **(Figure 3)**. In the food supplement industry,

524 the specific strain of a species is not always designated on the label, particularly for the generic, store-
525 brand, or less-established brands. This may be due in part to proprietary restrictions. We found that on
526 average, roughly half of the probiotics examined had the specific strain listed on the label, which varied
527 considerably by store (**Table 3).** Regulatory guidelines differ widely across different countries [66].
528 Probiotic labeling at the strain level is critical to so that consumers and/or healthcare providers can more
529 easily evaluate clinical studies of the probiotic's effects for specific indications [13], even if these
530 properties are shared among all members of a species [16]. This study of common commercially
531 available probiotics highlights the importance of supporting health claims by correctly identifying the
532 microbes in probiotics, and the importance of understanding the ecophysiological needs of a given
533 microbe to enable its beneficial effect (e.g. competition, colonization, flocculation, biofilm, antibiotic
534 production, adhesion, bioactive metabolite production). In the complex milieu of the digestive tract,
535 metabolic profiling of individual microbes and microbial communities can help draw the link between
536 prebiotics, probiotics, and overall digestive health. These insights could bring about strategies for
537 optimizing health and wellness, grounded in nutrition that promotes synergy with our commensal
538 microbiota.
539

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## Conflicts of Interest

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## References

1. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The Human Microbiome Project. Nature. 2007;449: 804–810. doi:10.1038/nature06244

2. Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. Genome Med. 2016;8: 51. doi:10.1186/s13073-016-0307-y

3. Reid G. The growth potential for dairy probiotics. Int Dairy J. Elsevier; 2015;49: 16–22. doi:10.1016/J.IDAIRYJ.2015.04.004

4. Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, Gómez-Llorente C, Gil A. Probiotic Mechanisms of Action. Ann Nutr Metab. 2012;61: 160–174. doi:10.1159/000342079

5. Borges S, Silva J, Teixeira P. The role of lactobacilli and probiotics in maintaining vaginal health. Arch Gynecol Obstet. 2014;289: 479–489. doi:10.1007/s00404-013-3064-9

6. Markowiak P, Śliżewska K. Effects of Probiotics, Prebiotics, and Synbiotics on Human Health. Nutrients. Multidisciplinary Digital Publishing Institute; 2017;9: 1021. doi:10.3390/nu9091021

7. Dahiya DK, Renuka, Puniya M, Shandilya UK, Dhewa T, Kumar N, et al. Gut Microbiota Modulation and Its Relationship with Obesity Using Prebiotic Fibers and Probiotics: A Review. Front Microbiol. Frontiers; 2017;8: 563. doi:10.3389/fmicb.2017.00563

8. Salazar N, Dewulf EM, Neyrinck AM, Bindels LB, Cani PD, Mahillon J, et al. Inulin-type fructans modulate intestinal Bifidobacterium species populations and decrease fecal short-chain fatty acids in obese women. Clin Nutr. 2015;34: 501–7. doi:10.1016/j.clnu.2014.06.001

9. Rodiño-Janeiro BK, Vicario M, Alonso-Cotoner C, Pascua-García R, Santos J. A Review of Microbiota and Irritable Bowel Syndrome: Future in Therapies. Adv Ther. 2018;35: 289–310. doi:10.1007/s12325-018-0673-5

10. Hungin APS, Mitchell CR, Whorwell P, Mulligan C, Cole O, Agréus L, et al. Systematic review: probiotics in the management of lower gastrointestinal symptoms - an updated evidence-based international consensus. Aliment Pharmacol Ther. Wiley/Blackwell (10.1111); 2018; doi:10.1111/apt.14539

11. Lee BJ, Bak Y-T. Irritable Bowel Syndrome, Gut Microbiota and Probiotics. J Neurogastroenterol Motil. Korean Society of Neurogastroenterology and Motility; 2011;17: 252–266. doi:10.5056/jnm.2011.17.3.252

12. Cai J, Zhao C, Du Y, Zhang Y, Zhao M, Zhao Q. Comparative efficacy and tolerability of probiotics for antibiotic-associated diarrhea: Systematic review with network meta-analysis. United Eur Gastroenterol J. 2018;6: 169–180. doi:10.1177/2050640617736987

13. Fijan S. Microorganisms with claimed probiotic properties: an overview of recent literature. Int J Environ Res Public Health. Multidisciplinary Digital Publishing Institute (MDPI); 2014;11: 4745–67. doi:10.3390/ijerph110504745

14. Nova E, Pérez de Heredia F, Gómez-Martínez S, Marcos A. The Role of Probiotics on the Microbiota: Effect on Obesity. Nutr Clin Pract. 2016;31: 387–400. doi:10.1177/0884533615620350

15. Elshaghabee FMF, Rokana N, Gulhane RD, Sharma C, Panwar H. Bacillus As Potential Probiotics: Status, Concerns, and Future Perspectives. Front Microbiol. 2017;8: 1490. doi:10.3389/fmicb.2017.01490

16. Sanders ME, Benson A, Lebeer S, Merenstein DJ, Klaenhammer TR. Shared mechanisms among probiotic taxa: implications for general probiotic claims. Curr Opin Biotechnol. 2018;49: 207–216. doi:10.1016/j.copbio.2017.09.007

17. Lenski RE. Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations. ISME J. 2017;11: 2181–2194. doi:10.1038/ismej.2017.69

18. Traverse CC, Mayo-Smith LM, Poltak SR, Cooper VS. Tangled bank of experimentally evolved Burkholderia biofilms reflects selection during chronic infections. Proc Natl Acad Sci. 2013;110: E250–E259. doi:10.1073/pnas.1207025110

19. Biolog. Gen III MicroPlate Instructions for Use. Hayward, CA. Available: www.biolog.com

20. Harley JP. Laboratory Exercises in Microbiology, Tenth Edition. New York, NY: McGraw-Hill Education; 2017.

21. Biolog I. Biolog MicroStation System/MicroLog User's Guide. Hayward, CA; 2010.

22. Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci U S A. 1985;82: 6955–6959. doi:10.1073/pnas.82.20.6955

23. Singhal N, Kumar M, Kanaujia PK, Virdi JS. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front Microbiol. 2015;6: 791. doi:10.3389/fmicb.2015.00791

24. Herbel SR, Vahjen W, Wieler LH, Guenther S. Timely approaches to identify probiotic species of the genus Lactobacillus. Gut Pathog. BioMed Central; 2013;5: 27. doi:10.1186/1757-4749-5-27

25. Dušková M, Šedo O, Kšicová K, Zdráhal Z, Karpíšková R. Identification of lactobacilli isolated from food by genotypic methods and MALDI-TOF MS. Int J Food Microbiol. 2012;159: 107–14. doi:10.1016/j.ijfoodmicro.2012.07.029

26. Sato J, Nakayama M, Tomita A, Sonoda T, Hasumi M, Miyamoto T. Evaluation of repetitive-PCR and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) for rapid strain typing of Bacillus coagulans. Natarajaseenivasan K, editor. PLoS One. Public Library of Science; 2017;12: e0186327. doi:10.1371/journal.pone.0186327

27. Kamboj K, Vasquez A, Balada-Llasat J-M. Identification and significance of Weissella species infections. Front Microbiol. Frontiers; 2015;6: 1204. doi:10.3389/fmicb.2015.01204

28. Larsen M V, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, et al. Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol. American Society for Microbiology; 2012;50: 1355–61. doi:10.1128/JCM.06094-11

29. Vancanneyt M, Huys G, Lefebvre K, Vankerckhoven V, Goossens H, Swings J. Intraspecific genotypic characterization of Lactobacillus rhamnosus strains intended for probiotic use and isolates of human origin. Appl Environ Microbiol. 2006;72: 5376–5383. doi:10.1128/AEM.00091-06

30. Di Cagno R, Minervini G, Sgarbi E, Lazzi C, Bernini V, Neviani E, et al. Comparison of phenotypic (Biolog System) and genotypic (random amplified polymorphic DNA-polymerase chain reaction, RAPD-PCR, and amplified fragment length polymorphism, AFLP) methods for typing Lactobacillus plantarum isolates from raw vegetables and fru. Int J Food Microbiol. 2010;143: 246–253. doi:10.1016/j.ijfoodmicro.2010.08.018

31. Ceapa C, Lambert J, van Limpt K, Wels M, Smokvina T, Knol J, et al. Correlation of Lactobacillus rhamnosus Genotypes and Carbohydrate Utilization Signatures Determined by Phenotype Profiling. Elkins CA, editor. Appl Environ Microbiol. 2015;81: 5458–70. doi:10.1128/AEM.00851-15

32. Kwong JC, McCallum N, Sintchenko V, Howden BP. Whole genome sequencing in clinical and public health microbiology. Pathology. 2015;47: 199–210. doi:10.1097/PAT.0000000000000235

33. Wuyts S, Wittouck S, De Boeck I, Allonsius CN, Pasolli E, Segata N, et al. Large-Scale Phylogenomics of the Lactobacillus casei Group Highlights Taxonomic Inconsistencies and Reveals Novel Clade-Associated Features. Dorrestein PC, editor. mSystems. 2017;2: e00061-17. doi:10.1128/mSystems.00061-17

34. Salvetti E, Harris HMB, Felis GE, O'Toole PW. Comparative genomics reveals robust phylogroups in the genus Lactobacillus as the basis for reclassification. Appl Environ Microbiol. American Society for Microbiology; 2018; AEM.00993-18. doi:10.1128/AEM.00993-18

35. Costea PI, Coelho LP, Sunagawa S, Munch R, Huerta-Cepas J, Forslund K, et al. Subspecies in the global human gut microbiome. Mol Syst Biol. European Molecular Biology Organization; 2017;13: 960. doi:10.15252/MSB.20177589

36. Truong DT, Tett A, Pasolli E, Huttenhower C, Segata N. Microbial strain-level population structure and genetic diversity from metagenomes. Genome Res. 2017;27: 626–638. doi:10.1101/gr.216242.116

37. Khatri I, Sharma S, Ramya TNC, Subramanian S. Complete Genomes of Bacillus coagulans S-lac and Bacillus subtilis TO-A JPC , Two Phylogenetically Distinct Probiotics. 2016; 1–25. doi:10.1371/journal.pone.0156745

38. Yao G, Gao P, Zhang W. Complete genome sequence of probiotic Bacillus coagulans HM-08: A potential lactic acid producer. J Biotechnol. 2016;228: 71–72. doi:10.1016/j.jbiotec.2016.04.045

39. Douillard FP, de Vos WM, Kleerebezem M, Vos W de, Purdom E, Francois F, et al. Functional

genomics of lactic acid bacteria: from food to health. Microb Cell Fact. BioMed Central; 2014;13: S8. doi:10.1186/1475-2859-13-S1-S8

40. Odamaki T, Horigome A, Sugahara H, Hashikura N, Minami J, Xiao J, et al. Comparative Genomics Revealed Genetic Diversity and Species/Strain-Level Differences in Carbohydrate Metabolism of Three Probiotic Bifidobacterial Species. Int J Genomics. 2015;2015: 1–12. doi:10.1155/2015/567809

41. Bottacini F, van Sinderen D, Ventura M. Omics of bifidobacteria: research and insights into their health-promoting activities. Biochem J. 2017;474: 4137–4152. doi:10.1042/BCJ20160756

42. Arboleya S, Bottacini F, O'Connell-Motherway M, Ryan CA, Ross RP, van Sinderen D, et al. Gene-trait matching across the Bifidobacterium longum pan-genome reveals considerable diversity in carbohydrate catabolism among human infant strains. BMC Genomics. BioMed Central; 2018;19: 33. doi:10.1186/s12864-017-4388-9

43. Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P, et al. Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pili containing a human-mucus binding protein. Proc Natl Acad Sci U S A. National Academy of Sciences; 2009;106: 17193–8. doi:10.1073/pnas.0908876106

44. Tytgat HLP, Douillard FP, Reunanen J, Rasinkangas P, Hendrickx APA, Laine PK, et al. Lactobacillus rhamnosus GG Outcompetes Enterococcus faecium via Mucus-Binding Pili: Evidence for a Novel and Heterospecific Probiotic Mechanism. Appl Environ Microbiol. American Society for Microbiology; 2016;82: 5756–62. doi:10.1128/AEM.01243-16

45. Buntin N, Hongpattarakere T, Ritari J, Douillard FP, Paulin L, Boeren S, et al. An Inducible Operon Is Involved in Inulin Utilization in Lactobacillus plantarum Strains, as Revealed by Comparative Proteogenomics and Metabolic Profiling. Elkins CA, editor. Appl Environ Microbiol. 2017;83: e02402-16. doi:10.1128/AEM.02402-16

46. Karam NE, Belarbi A. Detection of polygalacturonases and pectin esterases in lactic acid bacteria. World J Microbiol Biotechnol. 1995;11: 559–63. doi:10.1007/BF00286373

47. Nazzaro F, Fratianni F, Orlando P, Coppola R. Biochemical Traits, Survival and Biological Properties of the Probiotic Lactobacillus plantarum Grown in the Presence of Prebiotic Inulin and Pectin as Energy Source. Pharmaceuticals. 2012;5: 481–492. doi:10.3390/ph5050481

48. Goldstein EJC, Tyrrell KL, Citron DM. Lactobacillus Species: Taxonomic Complexity and Controversial Susceptibilities. Clin Infect Dis. Oxford University Press; 2015;60: S98–S107. doi:10.1093/cid/civ072

49. Meyer H, Weidmann H, Mäder U, Hecker M, Völker U, Lalk M. A time resolved metabolomics study: the influence of different carbon sources during growth and starvation of Bacillus subtilis. Mol Biosyst. 2014;10: 1812–23. doi:10.1039/c4mb00112e

50. Clerck E De, Rodriguez-diaz M, Forsyth G, Lebbe L, Logan NA, Devos P, et al. Polyphasic Characterization of Bacillus coagulans Strains , Illustrating Heterogeneity within this Species ,

and Emended Description of the Species. 2004;60: 50–60.

51. Linares DM, Gómez C, Renes E, Fresno JM, Tornadijo ME, Ross RP, et al. Lactic Acid Bacteria and Bifidobacteria with Potential to Design Natural Biofunctional Health-Promoting Dairy Foods. Front Microbiol. 2017;8: 1–11. doi:10.3389/fmicb.2017.00846

52. Campana R, van Hemert S, Baffone W. Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. Gut Pathog. BioMed Central; 2017;9: 12. doi:10.1186/s13099-017-0162-4

53. Herbel SR, Vahjen W, Wieler LH, Guenther S. Timely approaches to identify probiotic species of the genus Lactobacillus. Gut Pathog. 2013;5: 27. doi:10.1186/1757-4749-5-27

54. Menconi A, Kallapura G, Latorre JD, Morgan MJ, Pumford NR, Hargis BM, et al. Identification and characterization of lactic Acid bacteria in a commercial probiotic culture. Biosci microbiota, food Heal. IPEC, Inc.; 2014;33: 25–30. doi:10.12938/bmfh.33.25

55. Begovic J, Fira D, Terzic-Vidojevic A, Topisirovic L. Influence of carbohydrates on cell properties of Lactobacillus rhamnosus. Cent Eur J Biol. SP Versita; 2010;5: 103–110. doi:10.2478/s11535-009-0078-1

56. Celebioglu HU, Olesen SV, Prehn K, Lahtinen SJ, Brix S, Abou Hachem M, et al. Mucin- and carbohydrate-stimulated adhesion and subproteome changes of the probiotic bacterium Lactobacillus acidophilus NCFM. J Proteomics. 2017;163: 102–110. doi:10.1016/j.jprot.2017.05.015

57. Pan M, Kumaree KK, Shah NP. Physiological Changes of Surface Membrane in *Lactobacillus* with Prebiotics. J Food Sci. 2017;82: 744–750. doi:10.1111/1750-3841.13608

58. Kearney SM, Gibbons SM. Designing synbiotics for improved human health. Microb Biotechnol. Wiley/Blackwell (10.1111); 2018;11: 141–144. doi:10.1111/1751-7915.12885

59. Theilmann MC, Goh YJ, Nielsen KF, Klaenhammer TR, Barrangou R, Abou Hachem M. Lactobacillus acidophilus Metabolizes Dietary Plant Glucosides and Externalizes Their Bioactive Phytochemicals. MBio. American Society for Microbiology; 2017;8: e01421-17. doi:10.1128/mBio.01421-17

60. Tamang JP, Shin D-H, Jung S-J, Chae S-W. Functional Properties of Microorganisms in Fermented Foods. Front Microbiol. Frontiers; 2016;7: 578. doi:10.3389/fmicb.2016.00578

61. Derrien M, Veiga P. Rethinking Diet to Aid Human – Microbe Symbiosis. 2016;xx: 1–13.

62. Conlon MA, Bird AR. The impact of diet and lifestyle on gut microbiota and human health. Nutrients. Multidisciplinary Digital Publishing Institute (MDPI); 2014;7: 17–44. doi:10.3390/nu7010017

63. Zhao L, Zhang F, Ding X, Wu G, Lam YY, Wang X, et al. Gut bacteria selectively promoted by dietary fibers alleviate type 2 diabetes. Science (80- ). 2018;359: 1151–1156.

doi:10.1126/science.aao5774

64. Tramontano M, Andrejev S, Pruteanu M, Klünemann M, Kuhn M, Galardini M, et al. Nutritional preferences of human gut bacteria reveal their metabolic idiosyncrasies. Nat Microbiol. Nature Publishing Group; 2018;3: 514. doi:10.1038/s41564-018-0123-9

65. Lagier J-C, Dubourg G, Million M, Cadoret F, Bilen M, Fenollar F, et al. Culturing the human microbiota and culturomics. Nat Rev Microbiol. 2018; 1. doi:10.1038/s41579-018-0041-0

66. Arora M, Baldi A. Regulatory categories of probiotics across the globe: a review representing existing and recommended categorization. Indian J Med Microbiol. 2015;33 Suppl: 2–10. doi:10.4103/0255-0857.150868

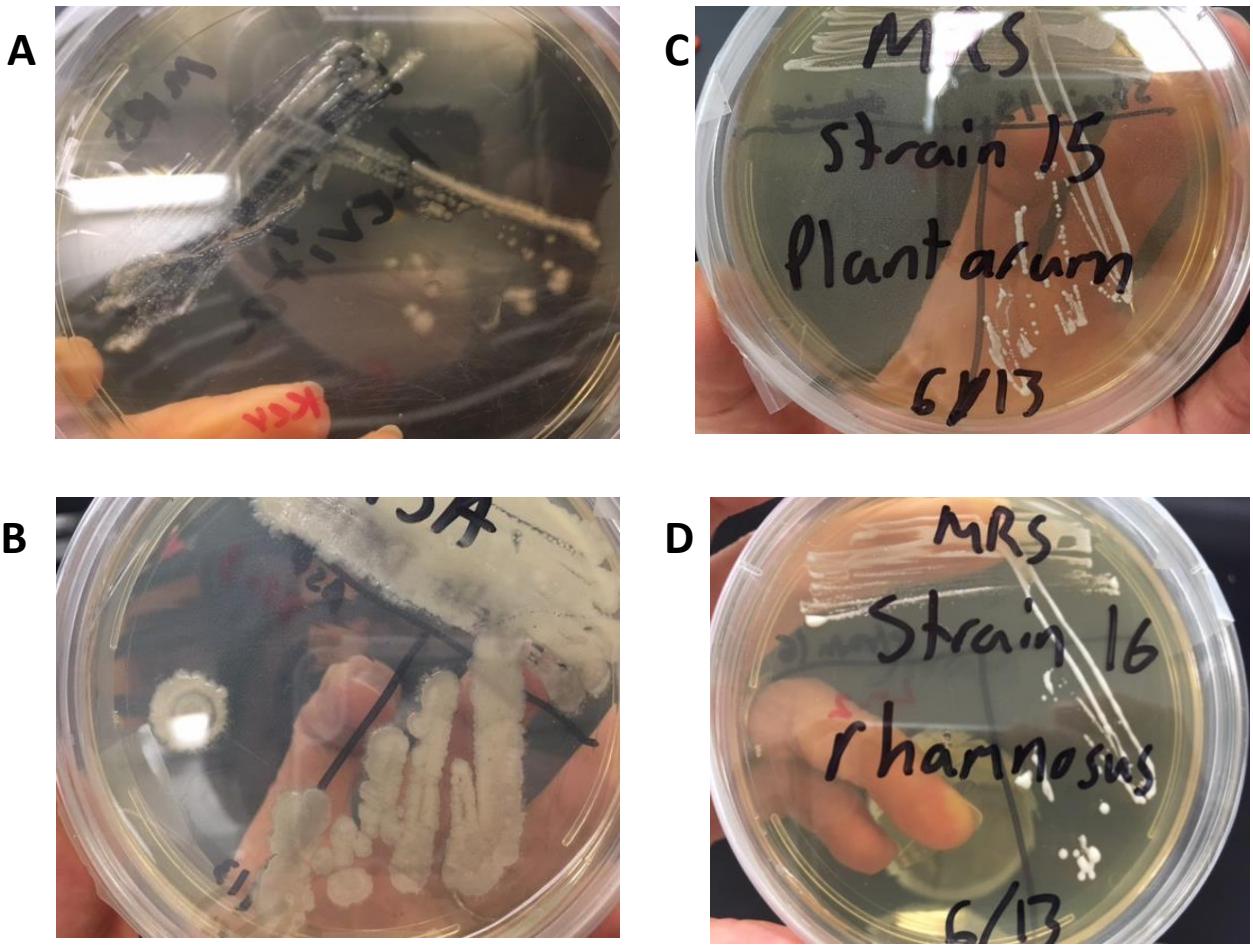



**Figure 1.** Typical colony morphology of probiotic bacterial colonies grown on agar media. A: *Bacillus coagulans* on MRS agar, B: *Bacillus subtilis* on TSA, C: *Lactobacillus plantarum* on MRS agar, D: *Lactobacillus rhamnosus*.on MRS agar.

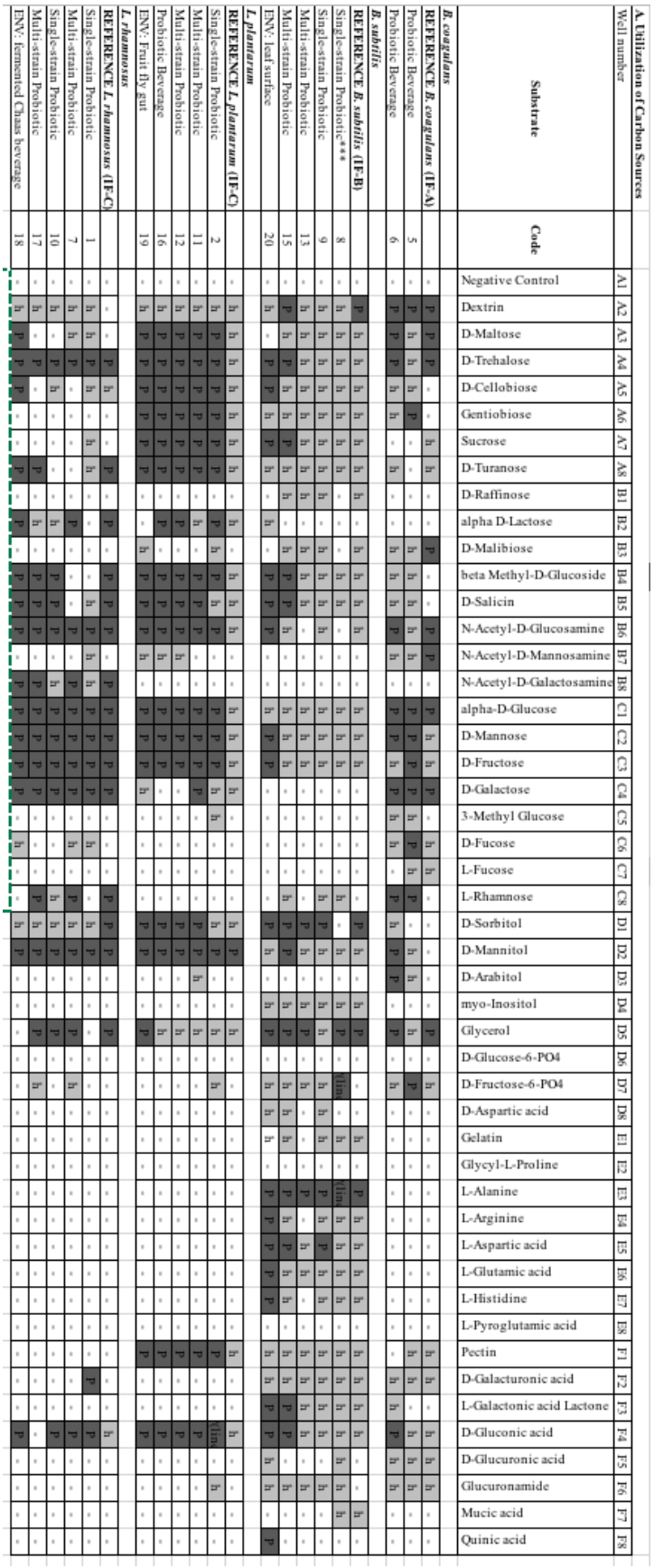



**Figure 2.** Phenotypic profiling of probiotic bacteria and yeast with Biolog system. P=positive, h=half (borderline positive/negative). **A**: Utilization of carbon sources. **B:** Tolerance to compounds, including antibiotic susceptibility. **C:** Carbon source utilization by yeasts isolated from probiotic drinks. Note that wells on the yeast (YT) plate in C contain different carbon sources than the plate for bacteria (GenIII). **D:** Example Biolog plate diagram showing the reference pattern for *Bacillus subtilis,* pink=positive; half-moon=borderline.

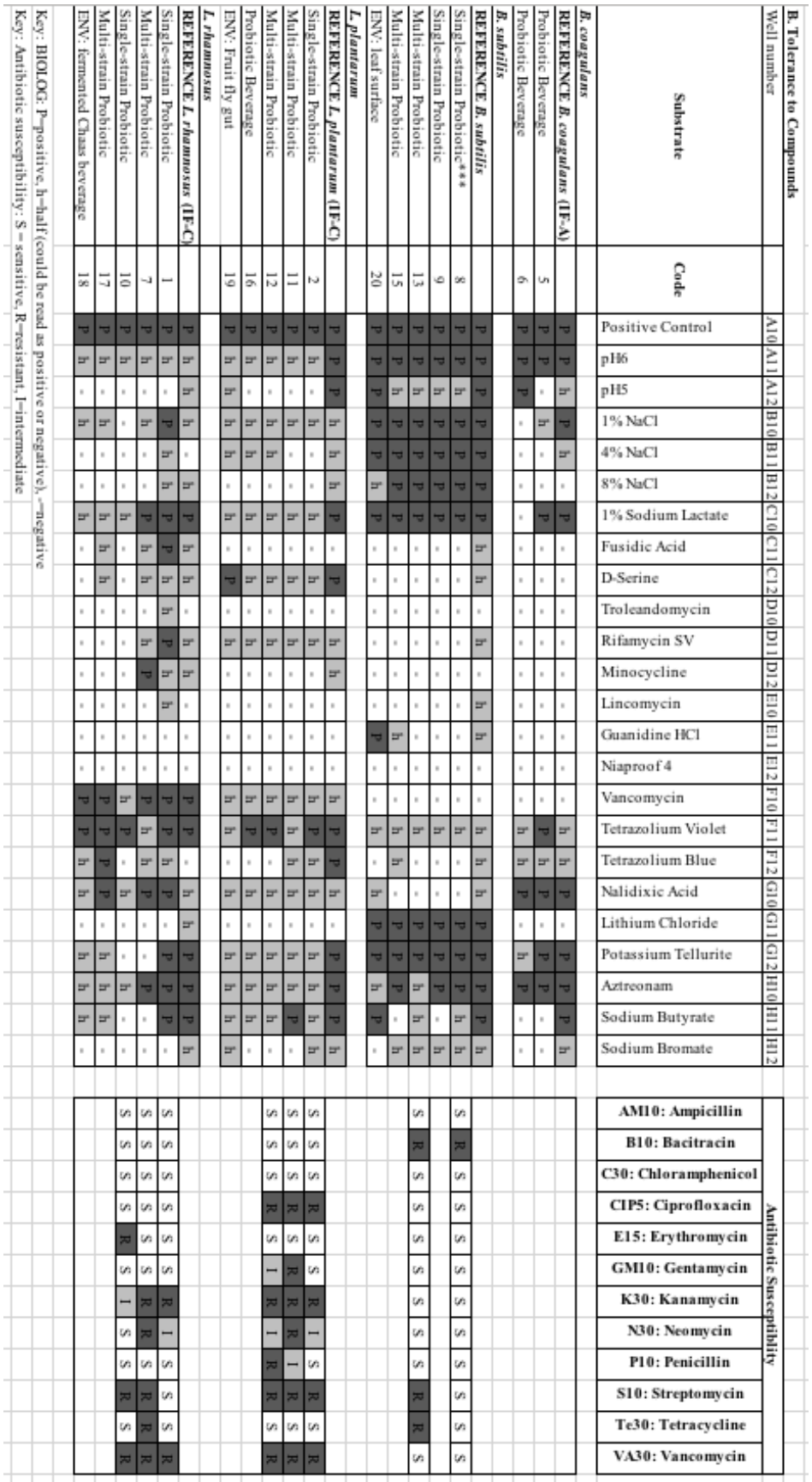


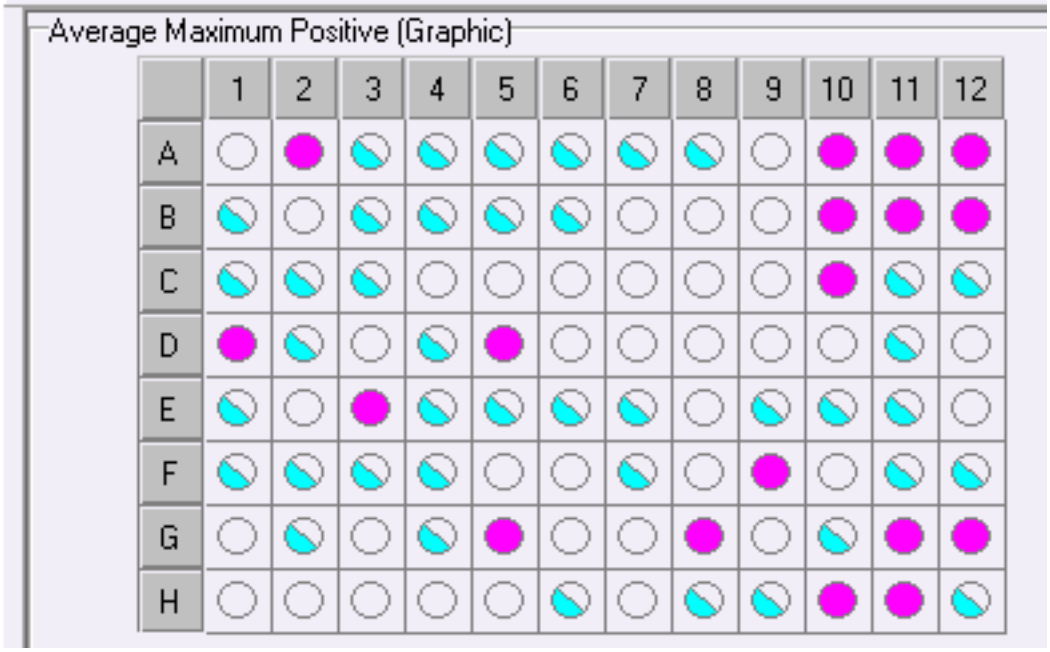

**Figure 2B.**

| Well number | | Substrate | Code | 21 | 22 | 25 | 24 | 23 | 26 |
|---|---|---|---|---|---|---|---|---|---|
| A1 | | Negative Control | | - | - | - | - | - | - |
| A2 | | Acidic acid | | - | - | - | - | - | - |
| A3 | | Formic Acid | | - | - | - | - | - | - |
| A4 | | Propionic Acid | | - | - | - | - | - | - |
| A5 | | Succinic Acid | | - | - | - | - | - | P |
| A6 | | Succinic Acid mono-methyl | | - | - | - | - | - | h |
| A7 | | L-Aspartic Acid | | - | - | - | - | - | - |
| A8 | | L-Glutamic Acid | | - | - | - | - | - | P |
| A9 | | L-Proine | | - | - | - | - | - | P |
| A10 | | D-Gluconic Acid | | - | - | - | - | h | h |
| A11 | | Dextrin | | - | - | - | - | - | P |
| A12 | | Inulin | | h | P | - | P | P | - |
| B1 | | D-Cellobiose | | - | - | - | - | P | P |
| B2 | | Gentobiose | | - | - | - | - | P | P |
| B3 | | Maltose | | P | P | P | P | - | h |
| B4 | | Maltoriose | | P | - | - | P | - | P |
| B5 | | D-Melezitose | | - | - | - | - | - | P |
| B6 | | D-Melibiose | | - | - | - | - | - | - |
| B7 | | Palatinose | | P | P | - | - | - | - |
| B8 | | D-Raffinose | | h | h | P | h | - | P |
| B9 | | Stachyose | | h | h | P | h | - | P |
| B10 | | Sucrose | | h | h | P | P | - | P |
| B11 | | D-Trehalose | | h | P | P | h | - | h |
| B12 | | Turanose | | P | P | P | P | - | - |
| C1 | | N-Acetyl-D-Glucosamine | | - | - | - | - | - | - |
| C2 | | alpha D-Glucose | | P | P | P | P | P | P |
| C3 | | D-Galactose | | P | P | - | P | - | h |
| C4 | | D-Psicose | | - | - | - | - | h | - |
| C5 | | L-Sorbose | | - | - | - | - | - | P |
| C6 | | Salicin | | - | - | - | - | P | P |
| C7 | | D-Mannitol | | - | - | - | - | - | P |
| C8 | | D-Sorbitol | | - | - | - | - | - | h |
| C9 | | D-Arabitol | | - | - | - | - | - | P |
| C10 | | Xylitol | | - | - | - | - | - | P |
| C11 | | Glycerol | | - | - | - | - | - | P |
| C12 | | Tween 80 | | - | - | - | - | - | - |

**Figure 2C.**

**Figure 2D**

| | |
|---|---|
| Organism Type | GP-Rod-SB |
| Family | Bacillaceae |
| Species | Bacillus subtilis ss subtilis |
| Protocol | B |

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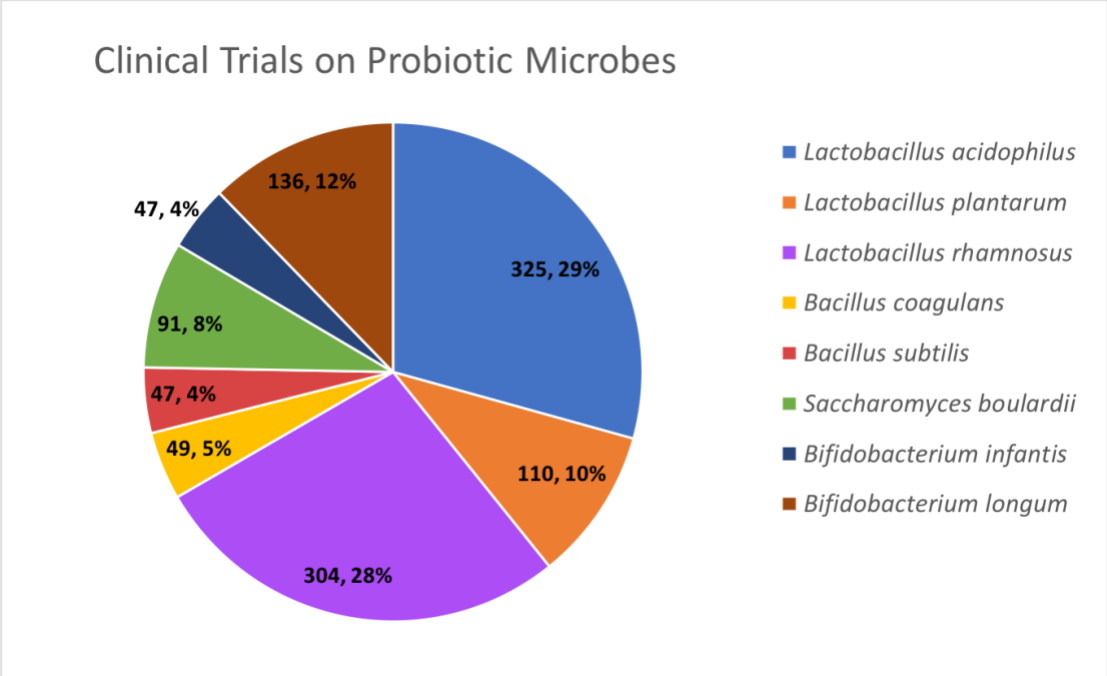


**Figure 3.** Number of published clinical trials on eight major probiotic species, cataloged in the Pubmed.gov database as of March 2018.