May 19, 2014

Determination of GRAS Status for *Bacillus subtilis*

In accordance with the FDA regulations governing GRAS status of food substances, 21 CFR §170.3 and §170.30, and the proposed regulations for GRAS notifications, 62 FR 18938 (17 April 1997), Deerland Enzymes has determined that *Bacillus subtilis subsp. inaquosorum str. DE111* is generally recognized as safe (GRAS), through scientific procedures including review of published scientific literature, and based on its common use in food.

This isolate of *Bacillus subtilis subsp. inaquosorum* was identified and designated as DE111 by Deerland Enzymes. DE111 is a dietary supplement and an ingredient for food for humans and other animals. The bacteria is present in the food supply and is offered at a level no higher than to achieve its intended purpose as a dietary supplement.

Included here is the result of the GRAS Panel Review, setting forth the basis for GRAS determination.

Sincerely,

Catherine Adams Hutt, PhD, RD
President, RdR Solutions, LLC
1. **Bacillus subtilis** subsp. *inaquosorum* str. **DE111** GRAS Determination

**Determination of GRAS Status Pursuant to Proposed 21 CFR §170.36 (C)(1)**

*Bacillus subtilis* subsp. *inaquosorum* str. **DE111** has been determined to be generally recognized as safe (GRAS) based on scientific procedures, including review of published scientific literature, and common use in food. The bacteria is found in the conventional food supply and have been consumed by a significant number of consumers for centuries. *Bacillus subtilis* subsp. *inaquosorum* str. **DE111** is being used as a dietary supplement at a level no higher than to achieve its intended purpose.

The basis for this finding is described in the following sections.

Signed,

[Signature]

Catherine Adams Hutt, PhD, RD

May 19, 2014

Agent for:

Deerland Enzymes
3800 Cobb International Blvd.
Kennesaw, GA 30152
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Expert Panel Statement

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1.0 GRAS Determination Information

1.1 Name and Address of Manufacturer

Deerland Enzymes
3800 Cobb International Blvd.
Kennesaw, GA 30152
Telephone: 404-409-5393

1.2 Common or Usual Name of the Substance

*Bacillus subtilis* subsp. *inaquosorum*

1.3 Conditions of Use

The intended use for DE111 is as a dietary supplement and an ingredient for food for humans and other animals. The supplement will be delivered in a powder blend: as a capsule or tablet, for addition to food, or other oral means in an amount no higher than to achieve its intended purpose, *i.e.*, no less than $10^6$ and no more than $10^{11}$ CFU/g.
1.4 Basis for GRAS Determination

DE111 has been determined to be GRAS by scientific procedures, including review of published scientific literature, and based on common use in food consumed by humans and other animals. Reference articles are identified in Appendix I.

Availability of Information

All information and data used to conduct this determination are publicly available. For additional information, please contact:

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2.0 Detailed Information about the Substance

Gram-positive Bacteria as a group are common soil organisms. *Bacillus* species are model mesophilic, aerobic heterotrophs that produce heat-resistant endospores. This isolate of *Bacillus* was isolated and enriched from a sample of soil and heated (80°C) to kill non-spore-forming mesophiles, and then plated on rich media and incubated aerobically at 30°C. Deerland Enzymes then identified it and designated this strain of *Bacillus subtilis* as DE111.

2.1 Identity

Whole genome sequence was obtained for the DE111. Genome-scale analysis showed that DE111 was a member of the *B. subtilis* subsp. *inaquosorum* group. DE111 was most closely related (98.7%) to the organism, *B. subtilis* subsp. *inaquosorum* (Rooney et al. 2009)

2.2 Method of Manufacture

Bacteria should always be grown from a single colony picked from a freshly streaked selective plate. Subculturing directly from glycerol stocks, agar stabs, and liquid cultures is poor microbiological practice. Inoculation from plates that have been stored for a long time may also lead to poor yield. The desired clone is streaked from a glycerol stock onto a freshly prepared agar plate containing the appropriate growth supporting ingredients such that single colonies can be isolated. This procedure is then repeated to ensure that a single colony of a clone can be picked. A single colony is inoculated into 2–10 mL of LB medium containing the appropriate selective agent and grown for ~8 hours (logarithmic phase). Using a vessel with a volume of at least four times greater than the volume of medium, the starter culture is then diluted 1/500 to 1/1000 into a larger volume of selective medium, and grown with vigorous shaking (~300 rpm) to saturation (12–16 hours).
General: Aerobic fermentation.

Growth Methods: Aerobic, 37°C, Neutral pH.

Liquid Culture Media:

Ingredients: cultures grown in standard Luria Bertani (Tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; NaCl, 10 g L^{-1}; pH 7.0) medium grown to a cell density of approximately $8 \times 10^{11}$ cells per mL.

Counting of Bacterial Particles:

One way to determine the minimum concentration of viable cells in culture is to exploit their ability to reproduce resulting in visible colonies on the surface of an appropriate nutritional agar in a Petri plate. The cells of this colony are a clones derived from the original cell (or small clump of cells) that were spread across the agar surface. The original cell (or small clump of cells) giving rise to the colony is called a colony forming unit (CFU). The concentration of bacteria is commonly expressed as the number of CFUs per volume (mL) or weight (g) of a sample. By counting colonies, a direct estimate of the concentration of viable bacteria or more accurately the number of colony forming units - CFU per mL in the original culture can be determined.

Calculation of Bacterial Yield:

To determine the number of CFUs per mL (CFU/mL) in a culture a small sample or aliquot of known volume is withdrawn from the culture and diluted into a known volume of sterile media. For example, one milliliter of a culture sample added to 9 milliliters of sterile media is equivalent to 1 part original culture to 10 parts diluted culture or a 1/10 dilution. These dilutions can be performed in series. Once diluted, the concentration of CFUs/mL can be determined in the diluted cell suspension. To do this, a measured volume of diluted culture, generally between 0.01
mL to 0.2 mL, is spread evenly over the surface of a LB-agar Petri plate. When incubated at 37 °C, the individual bacteria will grow up to form easily visible colonies within 18-24 hours. To calculate the concentration, the number of CFUs are divided by the volume plated and multiplied by the reciprocal of the dilution factor.

### 2.3 Specifications for Lots

Final product contents:

The final product contents is converted into CFU/g by dividing by the density of water (1 g/mL) to give a final concentration of $1 \times 10^6$ to $8 \times 10^{11}$ CFU/g.

### Quality Control Procedure:

DE111 used in our formulations has been thoroughly researched, documented, and banked in recognized microbial culture collections.

PCR identification by *gyrB*

Although 16S rRNA gene sequence analysis is the most frequently used method for identifying bacteria and constructing bacterial phylogenetic relationships its usefulness is limited. Identification of the 16S rRNA by PCR and subsequent analysis can identify the *B. subtilis* group, which includes different species (*B. subtilis, B. pumilus, B. mojavensis, B. vallismortis, B. atrophaeus, B. licheniformis, and B. amyloliquefaciens*). Use of 16s rRNA genes resolves to the level of genera, but is a poor tool for species and strain resolution. Genotypic approaches such as multi-locus sequence typing, or use of other house-keeping genes such as *gyrB* or *recA* are better at resolving species and strains. Experiments performed by Wang *et al.* (2007) showed that *gyrB* gene sequences provided higher resolution than 16S rRNA gene sequences and may be considered an efficient alternative target for identification analysis of members of the *B. subtilis*
group. PCR identification of DE111 is performed on each lot using the \textit{gyrB} primers BS-F (5’-GAAGGCGGNACNCAYG-3’) and bs-r (5’-CTTCRTGNGTNCCCGCTTC-3’). (Wang et al., 2007).

Rigorous testing program monitors stability throughout all manufacturing stages. Upon receipt, DE111 raw materials undergo independent laboratory analysis for verification of purity and stated potency prior to use in manufacturing.

DE111 formulations are manufactured and packaged at a GMP-compliant facility, where filtered air systems and a humidity and temperature controlled environment help ensure product stability and purity at this critical stage of handling.

To ensure that manufacturing and packaging processes do not adversely affect potency, all finished DE111 products are independently tested prior to release. DE111 enumeration, microbial content, and coliform content are evaluated on each batch to ensure quality of the final product.

Routine inclusion of higher quantities of microorganisms than stated on our labels coupled with refrigerated storage and cold-packaged shipping ensure that DE111, when properly stored and refrigerated, will retain their stated potency for at least 18 months from the date of manufacture. Shelf-life is confirmed by stability testing.

Detailed production records are maintained for all batches by Deerland Enzymes.

\textbf{Security:}

Deerland Enzymes is a secure facility containing company-owned incubators. The building has a secure key-card front door. Deerland Enzymes has confined office and laboratory spaces, which are also key-card secured. Refrigerators holding bacterial products are inside a laboratory space that is key locked at night.

\textbf{2.4 Bacillus subtilis Classification}

\textit{Bacillus subtilis} subsp. \textit{inaquosorum}
2.5 Identification, Potential Human and Other Animal Toxicants, Allergens, and Antibiotic Resistance

2.5.1 Bacterial Identification Analysis

Sequencing: Genomic DNA libraries were prepared using Illumina Nextera DNA kit. Whole genome sequence was performed on the Illumina MiSeq at the Cornell University Biotechnology Resource Center. The raw reads were assessed for quality by FastQC. Read size was predominantly 150 bp. The sequences were assembled by SPAdes 3.0. Scaffolds were evaluated for size and coverage with Sequencher. Scaffold sequences below 300 bp or 10-fold coverage were removed, leaving a total of 35 scaffold reads remaining.


**Results:**

DE111 was identified as a member of the *B. subtilis* subsp. *inaquosorum* group by 16S rRNA sequence analysis. Genome-scale comparative analysis for species identification against six reference *B. subtilis* subsp. *subtilis* genomes identified *B. subsp. inaquosorum*, as the closest relative (Table 1). Average nucleotide identity score (ANI) of 98.7% was above the cut-off score for species identification (>94.0%) indicating this isolate is a strain of *B. subtilis* subsp. *inaquosorum*. The genome size (4.32 Mbp) and GC content (43.9%) for the isolate was comparable for *B. subtilis* strains.
Table 1: Genome-scale Comparative Analysis for Species Identification

Results are provided in two files and can be opened using the web-based Artemis program [http://www.sanger.ac.uk/resources/software/artemis/](http://www.sanger.ac.uk/resources/software/artemis/).

“Bacillus_ordered_scaffold.fas” This file contains the genome content as individual assembled scaffold sequences that were aligned and ordered against the reference *B. subtilis* str. 168 genome. “Bacillus_pseudo.fas” This file contains the concatenated scaffold sequences into a single sequence. A gap marker, ‘NNN’, was inserted between scaffold sequences to enable evaluation of possible indels.

Genome size (Mb): 4.32

Coverage: 81x

No. of Scaffolds: 35

GC content (%): 43.9
Figure 1: Dendogram of Bacillus subtilis

* Indicates approximate location of Bacillus subtilis isolate based on genome-scale comparative analysis
2.5.2 Toxin Potential

Enterotoxin and Emetic Toxin Testing on DE111 by Polymerase Chain Reaction

There is no evidence of adverse effects from acute studies testing for toxins. DE111 was tested for genes responsible for the production of toxins, including haemolysin and lecithinase. No such genes were found.

*B. subtilis* produces different exo-enzymes contributing to the decay of organic matter. DE111 does not produce significant quantities of extracellular enzymes or toxins and is generally considered to have a low degree of virulence to humans.

Introduction:

Species within the genus *Bacillus* have been known to produce a variety of toxins. Testing has been done which identified strains outside the *B. cereus* group which had an ability to produce toxins. From Paik *et al.* (2005) found 8 toxin producing species of *Bacillus* out of 333 tested. The toxicity testing was done using *B. cereus* enterotoxin gene primers by polymerase chain reaction. The genome sequence was also examined for the presence of *B. cereus* enterotoxins to confirm the results of the polymerase chain reaction.

Materials and Method:

All testing was performed on an Applied Biosystems Step-one plus real time PCR, and all samples were prepped using a Qiagen DNeasy blood and tissue kit. Runs were completed using a modified version of the fast reaction base cycle setup. The samples were denatured at 95°C in an
initial holding step for a period of 2 minutes followed by 45 amplification cycles. The amplification cycles consisted of 15 seconds at 95°C, 30 seconds for annealing (temperature range of 43°C to 58°C dependent on the toxin being tested), and an elongation phase at 72°C for 30 seconds. Upon completion of the amplification cycles, a melt curve analysis was performed.

A sample of DE111 and a sample of Bacillus cereus, ATCC 10876, were prepped by initially plating cells diluted to an appropriate volume on nutrient agar and incubating for a period of 24 hours. After colonies were visible, approximately 3 were collected with a sterile loop and placed in 50mL of sterile nutrient broth a sterile centrifuge tube. The 50mL centrifuge tubes were then incubated in a shaker bath at 37°C for a period of 5-6 hours until an OD at 600nm of approximately 0.600 to 0.800 was reached when blanked with nutrient broth. One mL of Bacillus-rich broths were then transferred to sterile 2mL centrifuge tubes and spun down to pellet the bacteria. The supernatants were discarded, and the bacteria were resuspended in 180µL of lysis buffer. The enzymatic lysis buffer is a solution of Tric-HCl at 20mM (ph=8.0), 4mM EDTA, 1.2% Triton X-100, and 20mg/mL lysozyme added to an aliquot of the stock solution just prior to use. The microcentrifuge tubes of DE111 and lysis buffer and Bacillus cereus and lysis buffer were then incubated at 37°C for 30 minutes. After 30 minutes, 20µL of proteinase K and 200µL Buffer AL were added and the samples incubated at 56°C for 30 minutes. After the final incubation step, 200µL of 100% EtOH was added and the mixtures inverted several times to ensure homogeneity. Sample prep then followed the Qiagen purification of total DNA from animal tissues protocol. Furthermore, the DE111 was plated after incubating in the shaker bath to an OD of approximately 0.8 at 600nm to verify the concentration of cells present in the broth. Additionally, our strain of Bacillus subtilis was positively identified by a secondary outside lab.

Primers for the toxins Hemolysin B, Non-hemolytic enterotoxin A, B, and C as well as Cytotoxin K were used (From et al., 2005). A control primer for Bacillus subtilis was also used (Wang et al., 2007). All Primers were obtained through life technologies and diluted such that each PCR well contained approximately 200nM of forward and reverse primer. Overall reaction volume was 20µL containing 1µL forward and reverse primer, 6 µL nuclease free water, 2 µL sample prep, and 10 µL AB SYBR master mix. Toxins were run in sets based on ideal primer annealing temperatures (From et al., 2005). Bacillus cereus, ATCC 10876, was used as the positive control for the toxin primers.
Results:

Only one toxin amplification, hblC was observed during the 45 amplification cycles, as shown by the amplification plot in Figure 2. The control sample of DE111 amplified in all runs and a single product was verified using a melt curve analysis following each individual test. A negative control and a positive control (Bacillus cereus) were run for each toxin primer set as well as the Bacillus subtilis primers. No amplification was observed for any negative control across all test runs.

Based upon the genome sequence analysis, DE111 had no significant similarity with the B. cereus-like toxins Table 2.

Discussion:

Only one toxin displayed amplification within the 45 cycle pcr protocol. However, the genome sequence analysis found no similarity between hblC and DE111. Given the lack of amplification for any other toxin within the 45 cycle pcr protocol, we are confident the particular strain of Bacillus subtilis used in our testing does not contain the nucleotide sequences in question. To confirm the results of the PCR toxin screen, genome sequence was examined for the presence of B. cereus enterotoxins the results are displayed in Table 2. Additionally, DE111 amplified similarly in all testing and showed no tendency for primer dimer formation in the melt curve analysis. We saw amplification in the positive controls, and saw no amplification in the negative controls indicating that the primers chosen were acceptable for PCR analysis. All toxin tests were performed in a set dependent on the ideal annealing temperatures. This further strengthened our confidence in our results as we would expect the primers to run most efficiently at these values.
Figure 2. PCR amplification plot and well setup for toxin testing

<table>
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<th>Toxins</th>
<th>GenBank/Accession #/FASTA</th>
<th>Result</th>
<th>Query Covered</th>
<th>Identical</th>
</tr>
</thead>
<tbody>
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<td>hblC</td>
<td>BCU63928</td>
<td>No significant similarity found</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nheA</td>
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<td>No significant similarity found</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nheB</td>
<td>Y19005.2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>nheC</td>
<td>Y19005.3</td>
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<td></td>
<td></td>
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<tr>
<td>cytK</td>
<td>AJ277962.1</td>
<td>No significant similarity found</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Genome Sequence analysis for the presence of *B. cereus* enterotoxins

2.5.3 Allergen Potential

*Bacillus subtilis* subsp. *inaquosorum* str. DE111 proteins were analyzed against a database of known allergens at [www.allergenonline.org](http://www.allergenonline.org), no major allergens were identified. The AllergenOnline Database version 13 was accessed on February 12, 2013.

2.5.4 Antibiotic Resistance

ResFinder identifies acquired antimicrobial resistance genes in total or partial sequenced isolates of bacteria. DE111 was searched against a database of antibiotic resistance genes ([http://cge.cbs.dtu.dk/services/ResFinder/](http://cge.cbs.dtu.dk/services/ResFinder/)) and no antibiotic resistance genes were detected.
## ResFinder-2.1 Server – Results

<table>
<thead>
<tr>
<th>Class</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycoside</td>
<td>No resistance genes found.</td>
</tr>
<tr>
<td>Beta-lactam</td>
<td>No resistance genes found.</td>
</tr>
<tr>
<td>Fluoroquinolone</td>
<td>No resistance genes found.</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>No resistance genes found.</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>No resistance genes found.</td>
</tr>
<tr>
<td>MLS - Macrolide-Lincosamide-StreptograminB</td>
<td>No resistance genes found.</td>
</tr>
<tr>
<td>Nitroimidazole</td>
<td>No resistance genes found.</td>
</tr>
<tr>
<td>Phenicol</td>
<td>No resistance genes found.</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>No resistance genes found.</td>
</tr>
<tr>
<td>Sulphonomide</td>
<td>No resistance genes found.</td>
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<tr>
<td>Tetracycline</td>
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<tr>
<td>Trimethoprim</td>
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<tr>
<td>Glycopeptide</td>
<td>No resistance genes found.</td>
</tr>
</tbody>
</table>

**extended output**

Selected %ID threshold: **50.00 %**

Selected minimum length: **60 %**

Input Files: **DE111.fas**
Resistance to antibiotics targeting ribosomes, gyrases, or other enzymes and structures can occur with as little as a single nucleotide change in the chromosome of the bacterium. These changes are often overlooked when scanning sequences. To ensure the results from the ResFinder antibiotic screen did not overlook any changes, antibiotic susceptibility testing (AST) was performed. The guidelines and recommendations for the various antimicrobial testing methods, interpretive criteria, and QC parameters are established by the Clinical Laboratory Standards Institute (CLSI) Subcommittee on Antimicrobial Susceptibility Testing and published by the CLSI (http://www.clsi.org) (CLSI, 2012; CLSI, 2014). Because testing is done in different labs by different people, the guidelines standardize the conditions for testing such as inoculum size, growth medium and additives, incubation conditions and time and the antimicrobial concentrations. Several agar and broth dilution methods are available to test antibiotic effectiveness against a causative agent of disease. In this study, the Kirby-Bauer method was used to determine antibiotic susceptibility of DE111 (lot 08583) to twenty-two different antibiotics.

**Kirby-Bauer Method**

The Bauer, Kirby, Sherris and Turck method (Bauer et al., 1966), commonly called the Kirby-Bauer, Bauer-Kirby or filter disk diffusion method, allows microbiologists to test the

![Figure 3. Zones of inhibition around antibiotic disks by the Kirby-Bauer Method.](image)
effectiveness of an antibiotic against a bacterial species using paper disks containing a specific amount of an antibiotic. For non-fastidious bacteria, a standardized concentration of a test organism is inoculated onto a Mueller-Hinton agar plate. Then, a paper disk containing a specific antibiotic and amount is placed on the surface of the agar. These antibiotic disks are commercially available and are marked on their surface with the code to identify the antibiotic and amount. As the antibiotic dissolves in the moisture of the plate, it diffuses away from the disk to create an antibiotic concentration gradient. The concentration of the antibiotic in the agar decreases gradually as the distance from the disk increases. If the antibiotic is able to inhibit the growth of the organism, then a visible zone of inhibition develops around the disk after the plate has been incubated. The susceptibility or resistance of the bacterium to the antibiotic is determined by measuring the diameter (in millimeters) of the zone of inhibition. Once the measurement is taken, a table of performance standards is used to interpret the results (CLSI, 2014). For each antibiotic, the test organisms is reported as being susceptible (S), intermediate (I), or resistant (R) to the antibiotic.

Susceptible interpretation infers that the test organism is “inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used. The intermediate category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels, and for which response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated or when a higher than normal dosage of a drug can be used. And, the resistant category implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules, and/or that demonstrate MICs or zone diameters that fall in the range where specific microbial resistance mechanisms are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies” (CLSI, 2014).

Susceptibility of an infectious agent to an antibiotic is one factor to consider. The effectiveness of individual antibiotics varies with the location of the infection and the ability of the antibiotic to reach the site of infection. Allergy to an antibiotic, the effect on a fetus during pregnancy and other potential side effects also need to be considered.
AST Method

DE111 (lot 08583) was submitted by Deerland Enzymes, Inc for testing against twenty-four different antibiotics. AST protocols were followed as written in the CLSI’s Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Eleventh Edition, M02-A11 (CLSI, 2012) and briefly described below. The BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs (Becton Dickinson and Company) were used for to test all antibiotics with the exceptions of imipenem and quinupristin/dalfopristin, which used the Oxoid Antimicrobial Susceptibility Test Discs (Remel and Thermo Scientific). The antibiotics tested are listed below by class and include the disk code and amount in micrograms (or Units for penicillin). Testing of fusidic acid and nitroimidizoles (metronidazole, tinidazole) were not performed due to lack of availability of test disks.

**Aminoglycosides:**
- gentamicin (GM-120), kanamycin (K-30), neomycin (N-30), streptomycin (S-300)

**Beta-lactams- penicillins:**
- ampicillin (AM-10), amoxicillin/clavulanic acid (AmC-30), penicillin (P-10)

**Beta-lactams- cephalosporins:**
- cefaclor (CEC-30), cephalothin (CF-30), ceftriaxone (CRO-30), cefotaxime (CTX-30)

**Beta-lactams- carbapenems:**
- imipenem (IPM-10)

**Fluoroquinolone:**
- ciprofloxacin (CIP-5)

**Fosfomycin:**
- fosfomycin (FOS-200)

**MLS- Macrolide-Lincosamide-Streptogramin B**
- erythromycin (E-15), clindamycin (CC-2), quinupristin/dalfopristin (QD-15)

**Phenicol**
- chloramphenicol (C-30)

**Rifampicin**
- rifampin (RA-5)

**Sulphonamide:**
sulfamethoxazole-trimethoprim (SXT-23.75/1.25)

**Tetracycline:**
- tetracycline (Te-30)

**Trimethoprim (dihydrofolate reductase inhibitor):**
- trimethoprim (TMP-5)

**Glycopeptide:**
- vancomycin (Va-30)

*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as quality control test organisms according to CLSI protocols (CLSI, 2012, CLSI, 2014). *Escherichia coli* ATCC 35218, a known beta-lactamase producer, was used as an additional control organism when testing penicillin (P-10), ampicillin (AM-10) and amoxicillin/clavulanic acid (AmC-30). All bacteria used in this AST were maintained on Luria-Bertani (LB) agar plates and visually inspected for purity of culture. Twelve hours prior to testing, a single colony from the culture plate was aseptically transferred to a tube of LB broth and incubated at 35°C. Purity of culture was tested by aseptically streaking a loop-full of each broth culture onto a separate sterile LB agar plate using the streak plate method for isolation. The plates were examined the next day (after incubation at 35°C) for typical colony characteristics of the known bacterium without the presence of contaminating colonies.

One day prior to testing, Müeller-Hinton agar plates were prepared. The plates were incubated overnight at 35°C and visually inspected prior to use to ensure they were contamination free and did not have water condensation on the agar surface.

A **0.5 MacFarland Standard** is a turbidity standard comparable to a bacterial concentration of $1.5 \times 10^8$ CFU/ml. It is routinely used to adjust inoculum size for testing antibiotics and germicidal agents. The quality of the 0.5 MacFarland Standard is checked using matched cuvettes with a 1 cm light path and water as a blank standard. At a wavelength of 625 nm, the acceptable range for the turbidity standard is 0.08–0.13. A MacFarland standard was made by adding 0.5 mL of 0.048 M BaCl$_2$ (1.17% w/v BaCl$_2$·2H$_2$O) to 99.5 mL of 0.18 M H$_2$SO$_4$ (1% w/v) with constant stirring. After thoroughly mixing the McFarland standard to ensure even
suspension, 5 milliliters volumes were transferred to clear, screw capped test tubes and measured in a spectrophotometer for quality control.

A 0.5 MacFarland equivalent suspension for each bacterium was made by the transfer of 50 µl from the broth culture containing the bacterium to a tube of sterile, physiological saline (0.86% NaCl in deionized water w/v, 5 mL saline/tube). Once mixed, the suspension was compared to the MacFarland Standard for equivalent turbidity. The addition of 50 µl volumes of broth culture to the saline continued until the same turbidity of the standard was reached (generally requiring 250-500 µL of broth culture).

The Müeller-Hinton agar plates were inoculated by dipping a sterile cotton-tipped swab into the standardized suspension. The excess fluid was removed by pressing the cotton tip to the inside wall of the test tube. The surface of the test plates were inoculated using a confluent pattern that covered the entire surface area of the plate. The plates sat at room temperature for 5 minutes to ensure absorption of the suspension into the agar surface.

Antibiotic disks were aseptically applied onto the surface of the inoculated Müeller-Hinton plates. One disk was applied per quadrant; or, four antibiotic disks per plate. Sterile forceps were used to lightly tap the disks onto the agar. Plates were incubate the plates for 18 hours at 35°C.

The AST plates and purity of culture plates were removed from incubation and visually inspected for uniformity. If no irregularities were observed, a metric ruler was used to measure the diameter of the zone of inhibition (if present) around each antibiotic disk and the results recorded. When a zone of inhibition was present and measured, interpretation of the measurement required the use of the table on the manufacturer’s package insert (http://www.bd.com/ds/technicalCenter/inserts/8840621(201107).pdf) for the BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs or tables 2A-2J in the CLSI M-100-S24 (CLSI, 2014) for the Oxoid Antimicrobial Susceptibility Test Discs.

If no zone of inhibition was observed, a value of 6 mm equivalent to the diameter of the disk was recorded and interpreted as a resistant (R) result.
Results

Table 3. Measured diameters for the zones of inhibition around 23 different antibiotics for *Bacillus subtilis*. Measurement was interpreted as resistant, intermediate or susceptible to the antibiotic. Measurement of zones for control strains were interpreted as meeting the quality control range (Y = yes) or not (N = No). Blanks imply testing is not done or no control range has been published.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Zone of Inhibition (mm) / Interpretation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DE111$^a$</td>
</tr>
<tr>
<td><strong>Aminoglycosides</strong></td>
<td></td>
</tr>
<tr>
<td>Gentamicin, GM-120</td>
<td>36</td>
</tr>
<tr>
<td>Kanamycin, K-30</td>
<td>30</td>
</tr>
<tr>
<td>Neomycin, N-30</td>
<td>20</td>
</tr>
<tr>
<td>Streptomycin, S-300</td>
<td>19</td>
</tr>
<tr>
<td><strong>Beta-lactams- penicillins:</strong></td>
<td></td>
</tr>
<tr>
<td>Ampicillin, AM-10</td>
<td>26</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid, AmC-30</td>
<td>21</td>
</tr>
<tr>
<td>Penicillin, P-10</td>
<td>28</td>
</tr>
<tr>
<td><strong>Beta-lactams- cephalosporins:</strong></td>
<td></td>
</tr>
<tr>
<td>Cefaclor, CEC-30</td>
<td>28</td>
</tr>
<tr>
<td>Cephalothin, CF-30</td>
<td>27</td>
</tr>
<tr>
<td>Ceftriaxone, CRO-30</td>
<td>24</td>
</tr>
<tr>
<td>Cefotaxime, CTX-30</td>
<td>25</td>
</tr>
<tr>
<td><strong>Beta-lactams- carbapenems:</strong></td>
<td></td>
</tr>
<tr>
<td>Imipenem, IPM-10</td>
<td>48</td>
</tr>
<tr>
<td><strong>Fluoroquinolone:</strong></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin, CIP-5</td>
<td>30</td>
</tr>
<tr>
<td><strong>Fosfomycin:</strong></td>
<td></td>
</tr>
<tr>
<td>Fosfomycin, FOS-200</td>
<td>26</td>
</tr>
</tbody>
</table>
### Macrolide-Lincosamide-Streptogramin B

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Zone</th>
<th>S</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin, E-15</td>
<td>25</td>
<td>S</td>
<td>Y</td>
</tr>
<tr>
<td>Clindamycin, CC-2</td>
<td>26</td>
<td>S</td>
<td>Y</td>
</tr>
<tr>
<td>Quinupristin/dalfopristin, QD-15</td>
<td>20</td>
<td>S</td>
<td>Y</td>
</tr>
</tbody>
</table>

### Phenicol

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Zone</th>
<th>S</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol, C-30</td>
<td>24</td>
<td>S</td>
<td>Y</td>
</tr>
</tbody>
</table>

### Rifampicin

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Zone</th>
<th>S</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampin, RA-5</td>
<td>22</td>
<td>S</td>
<td>Y</td>
</tr>
</tbody>
</table>

### Sulphonamide

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Zone</th>
<th>S</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfamethoxazole-trimethoprim, SXT-23.75/1.25</td>
<td>32</td>
<td>S</td>
<td>Y</td>
</tr>
</tbody>
</table>

### Tetracycline:

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Zone</th>
<th>S</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline (Te-30)</td>
<td>30</td>
<td>S</td>
<td>Y</td>
</tr>
</tbody>
</table>

### Trimethoprim

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Zone</th>
<th>S</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimethoprim (TMP-5)</td>
<td>24</td>
<td>S</td>
<td>Y</td>
</tr>
</tbody>
</table>

### Glycopeptide:

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Zone</th>
<th>S</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin (Va-30)</td>
<td>21</td>
<td>S</td>
<td>Y</td>
</tr>
</tbody>
</table>

*aS = susceptible, I = intermediate, R = Resistant.

*bDiameter of zone of inhibition for control species and strains are within published range: Y = yes, N = No.

The results from the antibiotic susceptibility testing (AST) of DE111 shown on table 2 indicate the bacterium was clearly susceptible to nineteen of the twenty four antibiotics tested. In regards to the beta-lactam antibiotics ampicillin and penicillin, the results vary according to which Gram-positive bacterial group DE111 would be allied. Because *B. subtilis* is a Gram-positive bacterium, its cell wall would likely be more closely related to other Gram-positive bacteria including the enterococci, streptococci and staphylococci. The interpretations of susceptibility for these bacterial groups differ. Susceptibility of enterococci to ampicillin is interpreted by a zone of inhibition diameter ≥17 mm, and ≥ 24 mm for streptococci. However, the zone must be ≥29 mm for staphylococci to be considered susceptible to ampicillin. Therefore, the zone of 26
mm for DE111 was interpreted as S when using enterococci and streptococci standards, but R for the staphylococci standard. For ampicillin, the *Staphylococcus aureus* control produced a zone of inhibition of 24 mm, and was below the expected published range of 27-35 mm. This result did not change when the test was repeated. Therefore, there may be room for misinterpretation of the result. Similarly, the zone of 28 mm around the penicillin disk was considered a resistant result by the staphylococci standard (≤28 = R), but susceptible by enterococci (≥15) and streptococci (≥24) interpretations.

Although DE111 was clearly susceptible to 1\textsuperscript{st} (cefaclor) and 2\textsuperscript{nd} (cephalothin) generation cephalosporins, results were mixed for 3\textsuperscript{rd} generation cephalosporins (ceftriaxone, cefotaxime). When tested against ceftriaxone (CRO-30), a diameter of 24 mm was measured. This would be considered S for staphylococci (≥21 mm) and beta-hemolytic streptococci (≥ 24 mm), but R for viridans streptococci (≤24 mm). The zone of inhibition for cefotaxime (CTX-30) was 25 mm, which is interpreted as S for staphylococci (≥23 mm) and beta-hemolytic streptococci (≥ 24 mm), but R for viridans streptococci (≤25 mm). It was noticed that the zone of inhibition around each of the β-lactam cephalosporin disks was 5-10 mm smaller when compared to the *S. aureus* control. Phylogenetic analyses place *Bacillus subtilis* closer to the staphylococci than to the streptococci or enterococci (Ahmad et al., 2000); however, these analyses do not account for differences in antibiotic resistance or sensitivity mechanisms. The enhanced resistance of DE111 to β-lactams (in comparison to *S. aureus*) is not due to the expression of β-lactamase or resistance to penicillin and other β-lactam antibiotics would have occurred. Rather, the differences in antibiotic susceptibility are likely based on intrinsic factors such as accessibility to and interaction with the target proteins, such as transpeptidases, in the cell wall of *B. subtilis*. These results are in agreement with the negative results that were seen when polymerase chain reaction was used to amplify β-lactamase genes in this strain of *B. subtilis*.

Based on the zones around the remaining antibiotics, DE111 is considered susceptible to each. Two of the zones were within 3 mm of an intermediate interpretation including rifampin (RA-5) with a diameter of 22 mm (I is 17-19 mm) and quinupristin/dalfoprisitin (QD 15) which measured 20 mm (I is16-18). Again, these phenotypic results are in agreement with the negative results from the ResFinder genomic screen described earlier.
2.6 Subacute Toxicity Testing for *Bacillus subtilis* subsp. *inaquosorum* str. DE111 *in vivo*

Rat LD50 oral: > 5000 mg/kg bw  
(\(\sim 2.5 \times 10^{10}\) cfu/kg bw)  
Rat LD50 inhalation: > 0.63 mg/l air; 4 h  
(\(\sim 5 \times 10^{8}\) cfu/kg bw)  
Rabbit LD50 dermal > 2000 mg/kg bw  
(\(\sim 1 \times 10^{10}\) cfu/ kg bw)  
Skin sensitization (Buehler test): positive (R43)

It should be noted that when manufacturing the final product, *Bacillus subtilis* subsp. *inaquosorum* str. DE111, is mixed with a diluent to establish a concentration of \(1 \times 10^6\) to \(8 \times 10^{11}\) CFU/g because recommended oral administration provides no less than \(10^6\) and no more than \(10^{11}\) CFU/g. However for the purpose of the toxicity testing, uncut *Bacillus subtilis* subsp. *inaquosorum* str. DE111 was used.

2.7 Storage Conditions and Stability

**Recommended Storage Conditions for DE111:**  
-20°C to 37°C

**Bacterial Stability:**  
Labeled for 24 months at recommended storage temperature.

**Storage of Bacteria:**  
The bacterial stocks are stored at room temperature.
3.0 Specifications for DE111

Specifications follow USP recommendations for Dietary Supplements.

<table>
<thead>
<tr>
<th>Description</th>
<th>Suspension of <em>Bacillus subtilis</em> subsp. <em>inaquosorum</em> str. DE111 preparation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Fermentation derived</td>
</tr>
<tr>
<td>pH</td>
<td>6.0-8.0</td>
</tr>
<tr>
<td>Specific Gravity</td>
<td>1.05g/mL (+/- .05%)</td>
</tr>
</tbody>
</table>

**Heavy Metals:**

- Lead               <1ppm
- Arsenic            <1ppm
- Mercury            <1ppm
- Cadmium            <1ppm

**Microbiological Properties:**

- Yeast and Mold    <100/g
- Enterobacteriaceae <30/g
- Coliforms         <30/g
- *E. coli*          Negative in 10g sample
- *Salmonella*      Negative in 10g sample
- *Staph. aureus*   Negative in 10g sample

4.0 Basis for Determining GRAS Status

4.1 *Bacillus subtilis* Safety

4.1.1 General Safety of *Bacillus subtilis*

The safety of *B. subtilis* has been well established. This bacteria is ubiquitous in nature and are routinely consumed in food by humans. The abundance of the bacteria in the environment and the constant exposure of mammalian species to them support our natural tolerance for *Bacillus subtilis* and reflect their general safety.
Safety concerns include the potential presence of (a) *Bacillus cereus* exotoxin genes and virulence factors and (c) antibiotic resistance.

### 4.1.2 Safety of *Bacillus subtilis* subsp. *inaquosorum* str. DE111 in Healthy Human Subjects

#### Study Design

Forty-one subjects were recruited for participation and signed the informed consent approved by the Institutional Review Board for the Protection of Human Subjects, at the University of Wisconsin-La Crosse. This probiotic supplement study was performed in a randomized double-blind, placebo-controlled fashion with daily intake for an average of 20 days (range of 15-23 days).

Criteria for inclusion in the study were adult patients (aged ≥ 18 years at time of participation), no reported illnesses at the time of recruitment, and no reported use of antibiotics for seven days prior to recruitment. Subjects were excluded from the study if antibiotic use was reported at any point throughout the duration of the study.

#### Prior to Capsule Consumption

All subjects completed the provided gastrointestinal questionnaire to gauge initial gastrointestinal symptoms. At this time, subjects were each given a booklet containing: a copy of their informed consent, serving size of typical foods, food diary pages, Bristol stool charts, and bowel movement records. Subjects were instructed to utilize the serving size and Bristol stool charts to aid in food diary and bowel movement documentation, respectively.

#### Blood Sample

Trained phlebotomists used routine venipuncture procedures with serum separation tubes to collect blood samples. Each subject provided a 12-hour fasted blood sample of 15mL. Blood was allowed to clot for 20 minutes at room temperature. The collection tubes were spun at 2,500 rpm for 15 minutes, which allowed for serum separation. The serum was poured off into two analysis tubes and sent to Gundersen Lutheran Medical Center, La Crosse, WI, for further analysis (Table...
4). Samples were analyzed using a Cobas 6000 (Roche/Hitachi) automated clinical chemistry and immunoassay system.

<table>
<thead>
<tr>
<th>Albumin</th>
<th>ALP</th>
<th>ALT</th>
<th>AST</th>
<th>BUN</th>
<th>Calcium</th>
<th>Chloride</th>
<th>CO₂</th>
<th>Creatinine</th>
<th>Glucose</th>
<th>Potassium</th>
<th>Sodium</th>
<th>Bilirubin</th>
<th>Protein</th>
<th>Cholesterol</th>
<th>HDL</th>
<th>LDL</th>
<th>NonHDL</th>
<th>Triglycerid</th>
<th>CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

Table 4. Components of metabolic and lipid panels. ALP = Alkaline Phosphatase, ALT= Alanine Transaminase, AST = Aspartate Transaminase, BUN = Blood Urea Nitrogen, and CRP = C Reactive Protein, a substance that reflects acute stress.

**Capsule Consumption**

Subjects were instructed to take the assigned capsule once per day, with or without food. If a dose was missed, subjects were instructed to take two capsules the following day. Recurring incidences of missed doses were to be reported to the project leader; none were reported.

**Final Day of Capsule Consumption**

All subjects completed the provided gastrointestinal questionnaire to gauge final gastrointestinal symptoms. At this time, subjects handed in their completed booklets and were given compensation for the participation and completion of this study.
**Blood Sample**

Trained phlebotomists used routine venipuncture procedures with serum separation tubes to collect blood samples. Each subject provided a 12-hour fasted blood sample of 15mL. Blood was allowed to clot for 20 minutes at room temperature. The collection tubes were spun at 2,500 rpm for 15 minutes, which allowed for serum separation. The serum was poured off into two analysis tubes and sent to Gundersen Lutheran Medical Center, La Crosse, WI, for further analysis (Table 4). Samples were analyzed using a Cobas 6000 (Roche/Hitachi) automated clinical chemistry and immunoassay system.

**Statistical Analyses**

Statistical analysis using the general linear model and within subject’s factor of time (pre and post) and between subject’s factor of capsule type (DE111 and placebo) was conducted with SPSS Version 21.0 (IBM). Main effects of time and time by capsule interactions were considered significant at p<0.05.
Bowel Movement Record Averages

Figure 4 Average number of bowel movements per day between the probiotic and control groups. Subjects in the probiotic group had significantly more daily bowel movements (alpha ≤ 0.05; P = 0.015).

Figure 5 Average stool type per day between the probiotic and control groups. Stool types were based on the Bristol stool chart and did not change significantly in either group over time.
**Blood Parameters**

Table 5 Electrolyte Panel: Values are expressed as mean ± standard error of the mean

(† significant difference with respect to time).

<table>
<thead>
<tr>
<th>Group</th>
<th>Na⁺ Pre (135-146 mmol/L)</th>
<th>Na⁺ Post (135-146 mmol/L)</th>
<th>K⁺ Pre (3.4-5.0 mmol/L)</th>
<th>K⁺ Post (3.4-5.0 mmol/L)</th>
<th>Cl⁻ Pre (96-108 mmol/L)</th>
<th>Cl⁻ Post (96-108 mmol/L)</th>
<th>CO₂ Pre (22-29 mmol/L)</th>
<th>CO₂ Post (22-29 mmol/L)</th>
<th>'Ca²⁺ Pre (8.5-10.4 mg/dL)</th>
<th>'Ca²⁺ Post (8.5-10.4 mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis</td>
<td>138.6 ± 0.3</td>
<td>140.0 ± 0.4</td>
<td>4.11 ± 0.04</td>
<td>4.11 ± 0.05</td>
<td>101.6 ± 0.3</td>
<td>101.4 ± 0.3</td>
<td>25.1 ± 0.3</td>
<td>25.8 ± 0.3</td>
<td>9.27 ± 0.05</td>
<td>9.41 ± 0.06</td>
</tr>
<tr>
<td>Control</td>
<td>139.6 ± 0.4</td>
<td>140.5 ± 0.3</td>
<td>4.16 ± 0.07</td>
<td>4.13 ± 0.05</td>
<td>101.9 ± 0.3</td>
<td>102.1 ± 0.3</td>
<td>25.9 ± 0.3</td>
<td>26.7 ± 0.3</td>
<td>9.37 ± 0.06</td>
<td>9.49 ± 0.07</td>
</tr>
</tbody>
</table>
Table 6 BUN, Creatinine, Protein, Albumin & Glucose: Values are expressed as mean ± standard error of the mean (†† significant difference with respect to time by pill type).

<table>
<thead>
<tr>
<th>Group</th>
<th>BUN Pre (8-26 mg/dL)</th>
<th>BUN Post (8-26 mg/dL)</th>
<th>Creatinine Pre (0.6-1.1 mg/dL)</th>
<th>Creatinine Post (0.6-1.1 mg/dL)</th>
<th>Protein Pre (6.4-8.3 g/dL)</th>
<th>Protein Post (6.4-8.3 g/dL)</th>
<th>Albumin Pre (3.4-4.8 g/dL)</th>
<th>Albumin Post (3.4-4.8 g/dL)</th>
<th>††Glucose Pre (70-98 mg/dL)</th>
<th>††Glucose Post (70-98 mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis</td>
<td>12.8 ± 0.7</td>
<td>13.6 ± 0.8</td>
<td>0.90 ± 0.03</td>
<td>0.89 ± 0.02</td>
<td>7.05 ± 0.06</td>
<td>7.11 ± 0.09</td>
<td>4.59 ± 0.04</td>
<td>4.57 ± 0.06</td>
<td>91.0 ± 1.0</td>
<td>85.9 ± 1.4</td>
</tr>
<tr>
<td>Control</td>
<td>12.9 ± 0.8</td>
<td>13.1 ± 1.0</td>
<td>0.80 ± 0.02</td>
<td>0.80 ± 0.03</td>
<td>6.78 ± 0.09</td>
<td>6.87 ± 0.11</td>
<td>4.46 ± 0.06</td>
<td>4.51 ± 0.08</td>
<td>86.2 ± 1.3</td>
<td>85.9 ± 1.0</td>
</tr>
</tbody>
</table>
Table 7 Bilirubin, ALKP, AST & ALT: Values are expressed as mean ± standard error of the mean († significant difference with respect to time).

<table>
<thead>
<tr>
<th>Group</th>
<th>†Bilirubin Pre (0.1-1.3 mg/dL)</th>
<th>†Bilirubin Post (0.1-1.3 mg/dL)</th>
<th>ALKP Pre (IU/L)</th>
<th>ALKP Post (IU/L)</th>
<th>AST Pre (0-36 IU/L)</th>
<th>AST Post (0-36 IU/L)</th>
<th>ALT Pre (0-40 IU/L)</th>
<th>ALT Post (0-40 IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic</td>
<td>0.83 ± 0.09</td>
<td>0.68 ± 0.08</td>
<td>65.1 ± 4.7</td>
<td>62.7 ± 4.3</td>
<td>19.5 ± 1.4</td>
<td>18.3 ± 0.8</td>
<td>16.3 ± 0.8</td>
<td>14.8 ± 1.0</td>
</tr>
<tr>
<td>Control</td>
<td>0.51 ± 0.07</td>
<td>0.45 ± 0.04</td>
<td>63.8 ± 2.9</td>
<td>62.9 ± 3.1</td>
<td>21.6 ± 1.4</td>
<td>23.2 ± 2.9</td>
<td>15.6 ± 1.3</td>
<td>18.9 ± 3.5</td>
</tr>
</tbody>
</table>
Table 8 Lipid Panel: Values are expressed as mean ± standard error of the mean († significant difference with respect to time).

<table>
<thead>
<tr>
<th>Group</th>
<th>†Cholesterol Pre (&lt;200 mg/dL)</th>
<th>†Cholesterol Post (&lt;200 mg/dL)</th>
<th>†Triglyceride Pre (33-137 mg/dL)</th>
<th>†Triglyceride Post (33-137 mg/dL)</th>
<th>HDL Pre (40-60 mg/dL)</th>
<th>HDL Post (40-60 mg/dL)</th>
<th>†LDL Pre (&lt;130 mg/dL)</th>
<th>†LDL Post (&lt;130 mg/dL)</th>
<th>NON HDL Pre (LDL+30 mg/dL)</th>
<th>NON HDL Post (LDL+30 mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic</td>
<td>165.0 ± 7.0</td>
<td>169.4 ± 6.7</td>
<td>98.4 ± 15.3</td>
<td>97.9 ± 5.7</td>
<td>59.5 ± 3.3</td>
<td>60.1 ± 3.2</td>
<td>85.9 ± 5.6</td>
<td>89.6 ± 5.4</td>
<td>105.5 ± 7.0</td>
<td>104.3 ± 5.9</td>
</tr>
<tr>
<td>Control</td>
<td>160.0 ± 7.0</td>
<td>169.0 ± 6.7</td>
<td>87.5 ± 11.9</td>
<td>103.8 ± 7.6</td>
<td>59.5 ± 3.1</td>
<td>59.4 ± 2.9</td>
<td>83.1 ± 6.5</td>
<td>88.9 ± 5.9</td>
<td>100.6 ± 7.0</td>
<td>109.6 ± 6.5</td>
</tr>
</tbody>
</table>
Figure 6 C Reactive Protein (CRP): Non-stressed range for CRP was defined as $\geq 0.8$ mg/dL by the GLMC clinical lab.

Clinical Study Safety Conclusions

Daily ingestion of one capsule containing approximately $5 \times 10^9$ CFU/dose of DE111 was well tolerated in healthy young adults consuming their usual and variable diets, as reflected by blood levels of important biomarkers. Markers of systemic acceptance, such as CRP and liver enzymes remained within acceptable ranges and gastrointestinal symptoms and bowel habits, if anything, improved with probiotic capsule consumption. Though this study did not support a beneficial effect on lipid profile, probiotic consumption simply had no significant effect on blood lipids in this healthy largely normolipidemic population. Finally, consumption of DE111 in the manner described herein, may improve glucose tolerance, corroborating the findings of non-human animal in vivo and in vitro studies by Al-Salami et al. (2008) and Seung-Min et al. (2013), respectively. This probiotic may be a useful and safe dietary supplement for those with, or at risk, for metabolic syndrome and diabetes mellitus, barring some unforeseen interaction of the probiotic with these disease states.
4.1.3 Safety of *Bacillus subtilis* subsp. *inaquosorum* str. DE111

The safety of *Bacillus subtilis* subsp. *inaquosorum* str. DE111 is based on the results from the human clinical trial as well as its identification as *Bacillus subtilis*. Gene mapping of the organism proved it is not closely related to *Bacillus cereus*. Further, this *Bacillus subtilis* strain showed no *B. cereus*-like enterotoxin activity (described in Section 2.5.2). In addition, allergen (Section 2.5.3) and antibiotic resistance screen testing (Section 2.5.4) showed sensitivity to all antibiotics tested. Moreover, analysis of the safety panel conducted by the University of Wisconsin La-Crosse, did not identify any hazards or risks associated with ingesting *Bacillus subtilis* subsp. *inaquosorum* str. DE111 (Section 4.1.2).

4.1.4 Regulatory Recognition of *Bacillus subtilis* Products for Humans and Animals

There is precedence with regulatory recognition of GRAS status for other *Bacillus subtilis* products, and recognition by other official government entities. The following summarizes recognition by authoritative bodies:

- *Bacillus subtilis* R0179 included in Health Canada Natural Health Product Ingredient Database
- Evaluation of *Bacillus subtilis* R0179 in Healthy Young Adults – a clinical trial authorized by National Institutes of Health (NCT01802151) (http://clinicaltrials.gov/ct2/show/NCT01802151); considering efficacy with primary outcome using a questionnaire of gastrointestinal symptoms, and secondary outcome of microbial diversity in stool.
- *Bacillus subtilis* GB03 – recognized by Health Canada Pest Management Regulatory Authority (PMRA) as technical fungicide for seed treatment to suppress seed and root disease.
- *Bacillus subtilis* subsp. *natto* approved in Japan as FOSHU (Food for Specific Health Use)
- Species of Genus *Bacillus* are granted Qualified Presumption of Safety (QSP) by EFSA (http://www.efsa.europa.eu/fr/topics/topic/qps.htm?wtrl=01)

### 4.2 Toxicity Studies

It is well known that bacterial pathogenicity is strain-specific, so every bacterial strain that may be a probiotic should be tested individually. Pathogenic potential of some *Bacillus* strains is known, so the European Scientific Committee on Animal Nutrition proposed a scheme for the testing of toxin production in *Bacillus* bacteria intended for use as feed additives (EU, 2000). Several *Bacillus* strains - *B. subtilis* and *B. licheniformis* (Sorokulova *et al.*, 2008), *B. subtilis* and *B. indicus* (Hong *et al.*, 2008) were tested according to this scheme and showed no evidence of toxicity. Additional testing in animals, including acute and chronic toxicity studies, also indicated safety of these strains. *B. subtilis* RO179 was safe in vitro toxicity studies and in chronic oral toxicity challenges, performed in rats (Tompkins *et al.*, 2008). Safety of *B. coagulans* was demonstrated in acute and sub-chronic oral toxicity (Endres *et al.*, 2009) and in chronic one-year oral toxicity (Endres *et al.*, 2011). Results of these studies indicated that treatment of animals with *Bacillus* bacteria even in the high doses caused no signs of toxicity or any other adverse effects, related to tested cultures. Toxicity data, obtained for *Bacillus* strains (Sorokulova *et al.*, 2008) were in accordance with the safety records for *Lactobacillus* and *Bifidobacteria* (Sims, 1964).
4.2.1 Subacute Toxicity Literature

Chronic toxicity has been studied on the *Bacillus subtilis* strain with no signs of toxicity or histological changes in organs or tissue. Sorokulova *et al.* (2008) studied chronic toxicity in pigs, rabbits, and mice with no adverse effects. There were no differences in hematological indexes measured in blood from control and treated animals. Similar outcome was observed by Hong *et al.* (2008) in rabbits fed daily dose of $10^9$ spores of two strains of *Bacillus subtilis*, including Natto. No adverse effects were evident upon histological examination of visceral organs or tissues, and no differences noted between treated and control animals.

4.2.2 Acute Toxicity Literature

Acute toxicity has been evaluated for *Bacillus subtilis* and is reported in literature. Sorokulova *et al.* (2008) reported no treatment-related deaths mice orally administered 5 X $10^7$ to 2 X $10^{11}$ CFU/mouse. They found no ill effects in animals when *Bacillus subtilis* was administered either IP or IV even at the highest doses studied, and therefore concluded that the oral LD$_{50}$ was more than 2 X $10^{11}$ CFU. All animals were clinically healthy with no sign of diarrhea or other treatment-related illness. Hong *et al.* (2008) used a higher dose of 1 X $10^{12}$ CFU spores in guinea pigs, considered to be the most sensitive of laboratory animals. The animals showed no abnormalities and no significant differences vs controls. Histological examination of organs and tissues revealed no inflammation or pathological changes, and no differences in haematological indexes measured in blood from treated or control animals. Tompkins *et al.* (2008) studied mice fed *Bacillus subtilis* R0179 at 2 X $10^9$ CFU/kg body mass/day for 28 days, and found no variations in behavior, food consumption, body mass, or visible organ lesions upon post-mortem examination.

5.0 Functionality as a Dietary Supplement

According to the Food and Agriculture Organization (FAO), probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO, 2001) Probiotics are typically from *Lactobacillus* and *Bifidobacterium* species of bacteria. However, probiotics based on *Bacillus* strains are increasingly popular around the World (Mercenier *et al.*, 2003; Sanders *et al.*, 2003) and have been long used in Eastern Europe for their prophylactic and
therapeutic use against several gastrointestinal disorders (Sorokulova et al., 2008). *Bacillus* species play a significant role in the gut because of their high metabolic activity. They possess innate activity, producing peptides, enzymes that are lytic, amylolytic, pectinolytic, lypolitic, proteolytic, and cellulolytic; along with other substances that are antimicrobial, antifungal, and antiprotozoan (Sorokulova, 2013). They support healthy gut function and stimulate normal microflora for the gut. *Bacilli* also produce amino acids (Simmov, 1992) and vitamins (Walter & Bacher, 1977; Bentley & Meganathan, 1982). Some strains effectively degrade cholesterol *in vitro* (Kim et al., 2002) and reduce low-density lipoproteins, hepatic total cholesterol, and triglycerides after oral administration in animals (Paik et al., 2005).

*Bacilli* can also affect the immunological status of the host through expression of activation markers on lymphocytes in a dose-dependent manner (Caruso et al., 1993). *Bacillus subtilis* spores stimulated cytokine production in vitro and after oral administration in mice (Huang et al., 2008; Huang et al., 2013). Cultures of *B. subtilis* were used throughout the 1950s as an alternative medicine due to the immunostimulatory effects of its cell matter, which upon digestion has been found to significantly stimulate broad spectrum immune activity including activation of specific antibody IgM, IgG and IgA secretion and release of CpG dinucleotides inducing INF A/Y producing activity of leukocytes and cytokines important in the development of cytotoxicity towards tumor cells. (Shylakhovenko et al., 2003). It was marketed throughout America and Europe from 1946 as an immunostimulatory aid in the treatment of gut and urinary tract diseases such as Rotavirus and *Shigella* (Mazza, 1994).

### 6.0 *B. subtilis* in the Conventional Food Supply

Bacteria of the *Bacillus* species are among the most widespread microorganisms in nature. They are ubiquitous, found in soil (Garbeva et al., 2003) and water (Ivanova, 1999). *Bacillus* bacteria are included in the normal microflora of the gut in healthy adults (Hong et al., 2009) and children (Ellis-Pegler et al., 1975). The normal number of bacilli in the gut can reach $10^7$ CFU/g (Benno & Mitsuoka, 1986). They are resistant to acid and bile and maintain viability in the gut (Duc et al., 2003). Hong et al. (2009) compared the density of spores found in soil (~$10^6$ spores per
gram) to that found in human feces (~$10^4$ spores per gram). The number of spores found in the human gut is too high to be attributed solely to consumption through food contamination. Soil simply serves as a reservoir, suggesting that *B. subtilis* inhabits the gut and should be considered as a normal gut commensal.

Under strict anaerobic conditions, *Bacilli* can use nitrate as an electron acceptor and grow anaerobically (Hoffmann et al., 1995; Nakano & Hulett, 1997).

Humans are constantly exposed to the *Bacillus* species from the environment with no evidence of infections outbreak due to these bacteria (except *B. anthracis* and *B. cereus*) or apparent ill effects. Some cases of infection associated with “nonpathogenic” *Bacillus* species are described (Oggioni et al. 1998); but the frequency of such cases is low and comparable with the frequency of infections known for other bacteria of normal microbiota, such as *Lactobacillus* (Cukovic-Cavka et al. 2006) and *Bifido bacteria* (Borriello et al. 2003).

Given their ubiquitous nature, bacilli frequently find their way into food. *Bacillus* counts in wheat, grain, and wholemeal products are reported to be $10^6$ CFU/g (Rogers, 1978; Pepe et al., 2003). Due to the heat resistant nature of *Bacillus* spores, they often survive the baking process and are found in bread and baked foods (Sorokulova et al., 2003). Bacillus are often present in raw milk and remain after pasteurization, and can be the predominant microflora in pasteurized milk products (Pendurkar & Kulkami, 1990).

*Bacillus* bacteria have a long history of safe use in foods. Over a period of many centuries these bacteria have been used for preparation of alkaline-fermented foods (Wang J & Fung DYC, 1996). *Bacillus* species are the major microflora in soya beans and are responsible for their fermentation into soya food products and condiments (Ray et al., 2000; Inatsu et al., 2006). In Japan, a culture of *Bacillus subtilis* subsp. *natto* is used to produce Nattō, a popular food made by fermenting cooked soya beans (Katz & Demain, 1977). Nattō (なっとう or 納豆?) is a traditional Japanese food made from soybeans fermented with *Bacillus subtilis*. (Daily Press, 1992). "It is a traditional soybean breakfast food from northern Japan and it is called nattō .... As a breakfast food, nattō is usually served over steamed rice and mixed with mustard and soy sauce." In Japan nattō is most popular in the eastern regions, including Kantō, Tōhoku, and
Hokkaido. (Shurtleff, & Aoyagi, 2012). Nattō is occasionally used in other foods, such as nattō sushi, nattō toast, in miso soup, tamagoyaki, salad, as an ingredient in okonomiyaki, or even with spaghetti. Sometimes soybeans are crushed and fermented, which is called 'hikiwari nattō'.

Today's mass-produced Nattō is sold in small polystyrene containers. A typical package contains two, three, or occasionally four containers, each 40 to 50 g. One container typically complements a small bowl of rice.

Nattō has a different nutritional makeup from raw soy beans, losing Vitamin A and several other vitamins and minerals. However, the calorie content of nattō is lower than that of raw soy beans. While soy beans are highly nutritious, the greatest nutritional value is found in the bean's hard fiber. Nattō includes the benefits of nutritious soy and softer dietary fiber without the high sodium content present in many other soy products, notably in miso. Nattō contains no cholesterol and is a significant source of iron, calcium, magnesium, protein, potassium, vitamins B6, B2, E, K2 and more (Soy-beans.org, 2013). When Nattō is mixed with egg and eaten with rice, Japanese call the dish a perfectly nutritious meal, covering all nutritional needs. Many countries produce similar traditional soybean foods fermented with *Bacillus subtilis*, such as *shuidòuchi* (水豆豉) of China, *cheonggukjang* (청국장) of Korea, *thuanao* (ข้าวเน่า) of Thailand, *kinema* of Nepal and the Himalayan regions of West Bengal and Sikkim, *hawaijaar* of Manipur, *akhuni* of Nagaland, *piak* of Arunachal Pradesh, India. (Arora et al. 1991; Shurtleff & Aoyagi, 2012).

In addition certain West African bean products are fermented with the *Bacillus*, including *dawadawa, sumbala*, and *iru*, made from néré seeds or soybeans, and *ogiri*, made from sesame or melon seeds.

### 7.0 Level of Use

The amount offered as a dietary supplement is at a level no higher than to achieve its intended purpose. The recommended oral administration provides no less than $10^6$ and no more than $10^{11}$
CFU/g. This level of use is consistent with dietary exposure and with the safety recognition by regulatory authorities in Japan, Europe, and Canada.
EXPERT PANEL STATEMENT

Determination of GRAS Status for Bacillus subtilis

The undersigned, an independent panel of recognized experts (hereinafter referred to as an "Expert Panel"), qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested by Deerland Enzymes to determine the GRAS status of Bacillus subtilis intended for use as a dietary supplement. The scientific literature for safety and toxicity was made available to the Expert Panel. The Expert Panel independently evaluated these materials and other information deemed appropriate and important. Following their independent and critical review, the Expert Panel conferred and unanimously agreed to the decision described herein.

Expert Panel Statement of GRAS Determination

Signed,

[Signatures]

Dr. Charles P. Moran Jr. 12/12/2014

Dr. Paul Straight 12/10/2014

Theodore Hersh, M.D., AGAF, MACG 08/20/2014
Theodore Hersh, MD, AGAF, MACG, is a professor of medicine, emeritus, at Emory University School of Medicine in Atlanta, Georgia, with more than 30 years of academic, medical practice, and research experience as a gastroenterologist-hepatologist. He was a graduate of Harvard College and obtained his medical degree from Columbia University, New York, New York, and completed his postgraduate training at the Mayo Clinic, Rochester, Minnesota. He was awarded the Premier Physician Award by the Georgia chapter of the Crohn’s and Colitis Foundation and is the author and coauthor of more than 140 publications and 4 medical textbooks. He was the developer of the patented Thione Complex™, a powerful combination of antioxidants, enzymes and mineral.

Dr. Charles P. Moran Jr completed a B.S. in Biology from the University of South Carolina in 1972, followed by a Ph.D. in Genetics from University of North Carolina (Chapel Hill) (K.F. Bott, advisor), and a post-doctoral fellowship with R. Losick at Harvard University, Cambridge, MA, 1979-1982. He joined the faculty in the Department of Microbiology and Immunology at Emory University School of Medicine in December, 1982. Currently Dr. Moran is a Professor of Microbiology and Immunology, Emory University School of Medicine. He has served on several National Institutes of Health (NIH) and National Science Foundation (NSF) study sections including, NIH Microbial Physiology and Genetics Study Section (MBC-1), NIH Procaryotic Cell and Molecular Biology (PCMB) as Chair, and the Microbial Genetics Advisory Panel, National Science Foundation. He served as Editor of the Journal of Bacteriology for 10 years, and served on Editorial Boards of Applied and Environmental Microbiology, Research in Microbiology (Pasteur Institute), and Biochemica et Biophysica Acta. Honors include Elected Fellow in American Academy of Microbiology. Research areas include transcription in bacteria, and regulation of cell differentiation in bacteria. Major research accomplishments include discovery of $\sigma^H$, a secondary RNA polymerase sigma factor in the bacterium *Bacillus subtilis* required for the initiation of endospore formation, analysis of structure function relationships of RNA polymerase sigma factors, and their interaction with promoter DNA, characterization of role of a phosphorylated DNA binding protein, Spo0A, in activation of promoters used by two different forms of RNA polymerase, characterization of the role of two anti-sigma factors in regulating cell-type specific gene transcription, and identification and characterization of
morphogenic proteins that function during the assembly of the bacterial endospore coat. More recently studies of sub-cellular targeting of bacterial proteins and cell-cell signaling during endospore development lead to the discovery of an intercellular channel through which the mother cell nurtures the forespore. This work has been published in more than 100 research papers and review articles.

Dr. Paul Straight’s training and current research focus on how microbes regulate transitions in cellular physiology and metabolism in response to environmental inputs. As a graduate student at the University of Colorado, Boulder, he investigated a regulatory kinase that coordinates spore formation and chromosome segregation during starvation-induced meiosis in baker’s yeast, *Saccharomyces cerevisiae*. As a postdoctoral researcher at Harvard Medical School, he developed a model system to study molecular mechanisms of bacterial competition. The model system uses two species of soil bacteria, *Bacillus subtilis* and various *Streptomyces* species, to dissect the chemicals and enzymes these organisms use to compete with each other. Now Dr. Straight leads his own laboratory in the Biochemistry & Biophysics department at Texas A&M University. There his lab has further developed models of bacterial competition, in particular focusing on regulation and function of specialized metabolism. His lab collaborated on adapting technology of imaging mass spectrometry to study the spatio-temporal location of specialized metabolites in cultures of bacteria. This technique enables the lab to map networks of specialized metabolites that constitute the bacterial competitive repertoire. The lab has expanded their toolset to include bacterial genome sequencing, analysis, and transcriptomics in order to build a comprehensive view of the molecular functions required for competitive fitness of bacteria.
Appendix I: REFERENCES


Sorokulova IB (2013) Modern status and perspectives of *Bacillus* bacteria as probiotics. *J Prob Health* 1(4) http://dx.doi.org/10.4172/2329-8901.1000e106


