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RdR
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December 3, 2018

Dr. Dennis M. Keefe
Director, Office of Food Additive Safety
HFS-200
U.S. Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Keefe,

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), I am submitting this GRAS Notification as agent on behalf of Deerland Probiotics and Enzymes. We have determined that the *Bacillus subtilis* strain named DE111 is generally recognized as safe (GRAS), through scientific procedures including review of published scientific literature, and based on its common use in food.

The *Bacillus subtilis* strain named DE111 is virtually identical to that in the common Asian food natto, which is derived by fermentation of *Bacillus subtilis*. The probiotic bacteria strain has been fully researched and shown to be safe. Scientific literature includes acute and sub-acute testing in animals. Furthermore, testing of *Bacillus subtilis* DE111 has proven to have no toxic effects, including no cytotoxicity and free of enterotoxins; no antibiotic resistance or other adverse effects.

Literature also includes results of research conducted with infants, children, and elderly with no adverse effects; and three clinical trials performed by Deerland to prove efficacy have confirmed that *Bacillus subtilis* DE111 is safe with no adverse health effects reported.

We submit that *Bacillus subtilis* DE111 is a substance exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act based on the determination that it is GRAS. It's intended use is as a probiotic ingredient for food for humans and other animals. The bacteria are present in the food supply and is offered at a level no higher than to achieve its intended purpose.

Best regards,

Catherine Adams Hutt, PhD, RD, CFS
Agent for Deerland Enzymes and Deerland Probiotics

GENERALLY RECOGNIZED AS SAFE (GRAS) CONCLUSION FOR THE USE OF *BACILLUS SUBTILIS* DE111 IN FOODS

SUBMITTED BY:
Deerland Probiotics and Enzymes
3800 Cobb International Boulevard
Kennesaw, GA 30152
USA

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740-3835

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1.0 (GRAS Determination Information) Signed statements and certification

4568 Elm Bottom Circle
Aubrey, TX 76227

November 26, 2018

Determination of GRAS Status for *Bacillus subtilis* DE111

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, 81 FR 54959 (17 August 2016), Deerland Probiotics and Enzymes has determined that *Bacillus subtilis* DE111 is generally recognized as safe (GRAS), through scientific procedures including review of published scientific literature.

Bacillus subtilis DE111 is an ingredient for conventional food for humans. The bacterium is present in the food supply and is offered at a level no higher than to achieve its intended purpose as a probiotic food ingredient.

Included in this document is the result of the GRAS Panel Review, setting forth the basis for GRAS determination.

Sincerely,

(b) (6)

Catherine Adams Hutt, PhD, RD, CFS
President, RdR Solutions, LLC

1.1 Name and Address of Manufacturer

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

1.2 Common or Usual Name of the Substance

Bacillus subtilis DE111

1.3 Conditions of Use

Bacillus subtilis DE111 is a natural component of food for humans. Based on demonstration of safety through scientific procedures and supported by its history of safe use in food, the ingredient will be delivered in food as a probiotic dietary ingredient. *Bacillus subtilis* DE111 will be delivered in addition to food for adults in an amount no higher than to achieve its intended purpose, *i.e*., no less than 1 X 10^6 and no more than 1 X 10^{10} CFU/serving. For children, ages 2-12, it will be added to food at a level no higher than to achieve its intended purpose at 1 X 10^9 CFU/serving as a probiotic food ingredient. For infants, it will be added at a level no greater than 2 X 10^8 CFU/100 ml infant formula.

For adults and children, *Bacillus subtilis* DE111 may be added to: baked goods and baking mixes; alcoholic beverages; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fats and oils; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy and cough drops; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes*; sugar; and sweet sauces, toppings, and syrups.

**Bacillus subtilis DE111 is not intended for use in any product that would require additional review by USDA.*

1.4 Basis for GRAS Determination

Bacillus subtilis 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.

GRAS Determination

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

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

Signed,

(b) (6)

Catherine Adams Hutt, PhD, RD, CFS
Agent for:
Deerland Probiotics and Enzymes
3800 Cobb International Blvd.
Kennesaw, GA 30152

1.5 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 Identity, Method of Manufacture, Specifications and Physical or Technical Effect

2.1 Identity

Whole genome sequence was obtained for the *Bacillus subtilis* DE111 isolate. Genome-scale analysis showed the isolate was a member of the *Bacillus subtilis* subsp. *inaquosorum* group. Therefore, Bacillus *subtilis* DE111 isolate was most closely related (99%) to the organism *Bacillus subtilis* subsp. *inaquosorum* (Rooney *et al*. 2009).

2.2 Method of Manufacture

All fermentation runs begin from a frozen permanent to prevent genetic drift. Bacteria are grown from a single colony picked from a freshly streaked selective plate. The desired clone is streaked from a glycerol stock onto a freshly prepared agar plate containing the appropriate selective agent such that single colonies can be isolated. This procedure is then repeated to ensure that a single colony of a clone is 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 are grown in standard fermentation broth (i.e. protein, 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 X 10^{11} cells per mL.

Counting of Bacterial Particles:

One way to determine the minimum concentration of viable cells in a 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 clone of the original cell or small clump of cells and are 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, we get a direct estimate of the concentration of viable bacteria or more accurately the number of colony forming units - CFU per mL or gram - in the original culture.

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. Once diluted, the number of CFUs in a known volume of the diluted solution is determined. 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. 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 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 are converted into CFU/g by dividing by the density of water (1 g/mL) to give a final concentration of 1 X 10^6 up to 1 X 10^{11} CFU/g or (1 mg up to 1 gram).

Quality Control Procedure:

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

A rigorous testing program monitors stability throughout all manufacturing stages. Upon receipt, *Bacillus subtilis* DE111 raw materials undergo laboratory analysis for verification of purity and stated potency prior to use in manufacturing. *Bacillus subtilis* DE111 formulations are manufactured and packaged at a GMP-compliant facility, with filtered air systems and humidity- and temperature-controlled environment which help ensure product stability and purity.

All finished *Bacillus subtilis* DE111 products are tested prior to release. *Bacillus subtilis* DE111 enumeration, microbial content, and coliform content are evaluated for each batch to ensure quality of the final product (**Figure 1**). Shelf-life of *Bacillus subtilis* DE111 is confirmed by stability testing and will retain its potency for at least 24 months. (Appendix II)

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

Figure 1. Sample specification for *Bacillus subtilis* DE111 final product

Probiotic Count

Test Parameter	**Specification**	**Method**
Total CFU Count	≥100 Billion CFU/dose	3.80.308

Physical/Chemical

Test Parameter	**Specification**	**Method**
Color	Cream to dark tan	3.80.181
Texture	Granular powder	3.80.181
Odor	Strong fermentation	3.80.181
Lead	< 1 ppm	ICP-MS
Cadmium	< 1 ppm	ICP-MS
Mercury	< 1 ppm	ICP-MS
Arsenic	< 1 ppm	ICP-MS

Microbiological Standards

Test Parameter	**Specification**	**Method**
Yeast and Mold	≤ 300 CFU/gram	3.80.030
Coliforms	≤ 100 CFU/gram	3.80.029
Enterobacteriaceae	≤ 100 CFU/gram	3.80.061
E. coli	Negative	3.80.059
Salmonella	Negative	3.80.034
Staphylococcus aureus	Negative	3.80.060

2.4 Storage Conditions and Stability

Recommended Storage Conditions for *Bacillus subtilis*:

20-25°C

All Non-Liquid Preparations:

Store at room temperature (20-25°C), in an airtight container, away from light and moisture. *Bacillus subtilis* DE111 viability was consistent at 25 ± 2°C with 40 ± 5% RH over the course of 24 months (Appendix II).

Bacterial Stability:

Labeled for 24 months at recommended storage temperature.

Storage of Bacteria:

The bacteria are stored at recommended storage temperatures.

2.4.1 Shelf Stability of *Bacillus subtilis* DE111 for 24 Months

Bacillus subtilis DE111 is stable over 24 months under manufacturer recommended storage conditions. No loss was detected within the range of the assay performed. (Appendix II)

2.5 Survivability

Total *Bacillus subtilis* DE111 does not reduce viability / concentration after contact with acidic or salt concentrated nutrient broth for 24 hours. Additionally, *Bacillus subtilis* DE111 is not sensitive to acid and bile and it is capable of maintaining viability in low pH and high salt concentrations (Appendix III).

2.6 Bacterial Classification

Bacillus subtilis subsp. *inaquosorum*

2.7 Bacterial Identity, Potential Human and Other Animal Toxicants, Allergens, and Antibiotic Resistance

2.7.1 Bacterial Identification Analysis

Sequencing and bioinformatics methods identified the *Bacillus subtilis* DE111 isolate as a member of the *Bacillus subtilis* subsp. *inaquosorum* group by 16S rRNA sequence analysis.

Average nucleotide identity score (ANI) of 99 % was above the cut-off score for species identification (>94.0%) indicating the *Bacillus subtilis* DE111 isolate is a strain of *Bacillus subtilis* subsp. *inaquosorum*. The genome size (4.32 MBP) and GC content (43.9%) for the isolate was comparable for *Bacillus subtilis* strains (Appendix IV).

2.7.2 Enterotoxin and Emetic Toxin Testing on *Bacillus subtilis* DE111 by Polymerase Chain Reaction

There is no evidence of adverse effects from acute studies testing for toxins in strains of *Bacillus subtilis*. *Bacillus subtilis* DE111 was tested for genes responsible for the production of toxins common in pathogenic strains such as *Bacillus cereus*. Real time PCR was performed using purified DNA from *Bacillus subtilis* DE111 and primers for the toxins including Hemolysin B, Non-hemolytic enterotoxin A, B, and C as well as Cytotoxin K to evaluate for amplification. Furthermore, upon genome sequence analysis, *Bacillus subtilis* DE111 was found to not have significant similiarity with the *Bacillus cereus*-like toxins including hb1C, nheA, nheB, nheC, and cytK (Appendix V)**.** *Bacillus subtilis* DE111 does not produce toxins and does not contain any deleterious genes. *Bacillus subtilis* DE111 is considered a non-virulent organism to humans.

2.7.3 ALLERGEN POTENTIAL

Bacillus subtilis DE111 has not come into contact with a priority allergen or derivative (e.g. soy, gluten, milk, fish via the culture media) (list available at: *http://www.hc-sc.gc.ca/fn-an/securit/allerg/fa-aa/index-eng.php*). The complete genome sequence of *Bacillus subtilis* DE111 (http://www.ncbi.nlm.nih.gov/nuccore/CP013984.1) proteins were analyzed against a database of known allergens at www.allergenonline.org; no major allergens were identified (Appendix XIII).

2.7.4 ANTIBIOTIC RESISTANCE

ResFinder is a tool that identifies acquired antimicrobial resistance genes in total or partial sequenced isolates of bacteria. *Bacillus subtilis* DE111 was searched against a database of antibiotics (http://cge.cbs.dtu.dk/services/ResFinder/) and no antibiotic resistance genes were detected. (Appendix VI)

Additionally, antimicrobial susceptibility of *Bacillus subtilis* DE111 was assessed using the zone of inhibition method and minimal inhibitory concentration micro-dilution assays. Both methods showed that *Bacillus subtilis* DE111 is susceptible to common antibiotics showing no antimicrobial resistance genes (Appendix VII, Appendix VIII). Research indicates that *Bacillus subtilis* DE111 risk of antibiotic resistance gene transfer is extremely low.

2.8 SUBACUTE TOXICITY TESTING FOR *BACILLUS SUBTILIS IN VIVO*

Rat LD50 oral: > 5000 mg/kg bw ; 35 days

(~ 2.5 x 10^{10} cfu/kg bw)

Rat LD50 inhalation: > 0.63 mg/l air; 4 h

(~ 5 x 10^{8} cfu/kg bw)

Rabbit LD50 dermal > 2000 mg/kg bw

(~ 1 x 10^{10} cfu/ kg bw)

Skin sensitization (Buehler test): positive (R43)

"It should be noted that when manufacturing the final product, *Bacillus subtilis*, is mixed with a diluent to establish a concentration of 10^{6} to 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, the most concentrated form of *Bacillus subtilis* was used" (European Commision, 2006).

3.0 DIETARY EXPOSURE

3.1 HISTORY OF SAFE USE

Bacillus subtilis is widely used in different types of fermented foods. As an established ingredient in the preparation of several traditional fermented foods, *Bacillus subtilis* has a long history of consumption in Southeast Asia (*e.g*., natto) and Africa (*e.g*., ogiri) at levels up to 10^{11} CFU/g (Nout, 2015, Wang and Fung, 1996). *Bacillus subtilis* DE111 is virtually identical to that found in the food natto (Kubo et al, 2011). Research reports natto to contain 1 X 10^9 CFU *Bacillus subtilis* per gram (Homma et al, 2006). The USDA nutrient databank (USDA, SR-28) reports a serving of natto is 175 grams. Therefore, an individual consuming natto is consuming 1.75 X 10^{11} CFU *Bacillus subtilis* per serving/per day, given one serving of natto consumed per day. Since *Bacillus subtilis* bacteria has long been considered safe and suitable for human consumption, several published studies have addressed its safety (Fijan, 2014). *Bacillus subtilis* is a versatile bacterium that has been isolated from a range of environmental niches; for example, it has been shown to survive and colonize within the gastrointestinal tract of humans and other mammals (Tam et al., 2006). *Bacillus subtilis* strains isolated from fermented products have been demonstrated to produce bacteriocins that effectively prevent growth of pathogens, such as *Listeria monocytogenes* (Charles et al., 2007). Therefore, *Bacillus subtilis* may be considered a probiotic of safe consumption providing benefit to the host.

Toxicity in Animals

The safety of various strains of *Bacillus subtilis* has been reported in numerous animal studies and *in vitro* tests. These studies have been used to identify a level for which no adverse effects have been observed (NOEL). A representative selection of such studies is described below, with additional studies demonstrating safety appearing in section 6 and section 7 of this document.

Studies have been published showing the levels of dietary intake that failed to elicit adverse toxic effects. In one study, six female Sprague-Dawley rats were dosed once by gavage with *Bacillus subtilis*, at 2000 mg/kg in an acute oral toxicity study (Richeux, 2011). Treatment did not elicit clinical signs or deaths. Body weight gains were normal. Necropsy did not find treatment effects. LD50 was assessed at greater than 2000 mg/kg. Another study evaluated 90-day sub-chronic toxicity of *Bacillus subtilis* that was performed in both sexes of F344 rats by feeding of CRF-1 pellet diet containing 0%, 0.18%, 0.55%, 1.66% and 5% (Nakamura et al., 1999). Rats were randomly allocated to 5 groups, each consisting of 10 males and females. No animals died during the administration period and no differences in body weights and food intakes were found among groups of either sex. Kidney weight was significantly increased in both sexes in the groups given concentrations of 1.66% or more. However, the increases of kidney weight were slight in themselves and other data on serum biochemistry and histopathology did not show any apparent toxicological signs, including renal toxicity. These findings indicate that feeding *Bacillus subtilis* in the diet for 90 days does not create toxicity in rats even at the highest dose of 5% of the diet. Another study was carried out to investigate the acute *in vivo* single-dose toxicity of *Bacillus subtilis*, a probiotic candidate showing strong and broad antibacterial activity (Kyoung-Hoon et al., 2015). The test sample was orally administrated to male and female ICR mice at a highest dose of 2,000 mg/kg for 14 days. No significant change in general conditions, mortalities, body weight changes, clinical signs, autopsy findings, or presence of gross lesions was observed in either sex of mice. The results indicate that up to 2,000 mg/kg of *Bacillus subtilis* has no adverse effect on ICR mice.

Treatment dose effect was evaluated in a study using two hundred and twenty male BALB/c mice, 6–8 weeks of age, housed ten animals per cage. Different doses of *Bacillus subtilis* were administrated orally (5 X 10^7 to 2 X 10^{11} CFU/mouse), intraperitoneal (IP) and intravenous (IV) (5 X 10^7 to 5 X 10^9

CFU/mouse) to the BALB/c mice. There were no treatment-related deaths, even in groups of animals IP and IV treated with the *Bacillus* strains at the highest doses. Thus, the oral LD50 for the tested strains was more than 2 X 10^{11} CFU. LD50 for IP and IV-administrated *Bacillus subtilis* strains was more than 5 X 10^{9} CFU. The administration of *Bacillus subtilis* did not show any potential adverse effect on mouse activity and weight. All animals were clinically healthy, *i.e.*, no diarrhea or other treatment-related illness was recorded. There were no differences in the appearance of visceral organs between experimental and control groups of animals during macroscopic examination. On day 7 after the probiotic inoculations the spleen weight index (SWI) was measured for mice in the groups that were orally inoculated with the 5 X 10^{9} CFU of *Bacillus* probiotic strains and compared to the placebo control. No significant difference in SWI was observed between the groups. Microscopic observation found no signs of inflammation or any other pathological changes in all analyzed organs and tissues (Sorokulova et al., 2007).

Based on the available data using animal testing, the no-observed-adverse-effect level (NOAEL) for *Bacillus subtilis* in male and female rats is 2000 mg/kg/day (equivalent to 2 X 10^{11} CFU/kg bw/day), the highest dose evaluated.

It is understood that while short-term or acute studies were considered adequate even for major food additives several decades ago, today's recommendations generally include comprehensive, long-term toxicity studies (FDA Redbook, 2007). We further understand that CFSAN toxicologists exercise their best scientific judgement in determining what toxicity studies are needed for the Agency to adequately assess the safety of a direct food additive or color additive used in food (FDA Redbook, 2007). In making these decisions, it is expected that toxicologists consider what is already known about the properties of a compound, its intended conditions of use, and current standards for toxicity testing.

Cytotoxicity

In addition to assessing the safety of food additives through animal studies, numerous *in vitro* alternatives have been accepted by regulatory agencies in lieu of animal studies; including genetic toxicity and human cell line testing (NTP, 2016; OECD, 2015a; OECD, 2015b; OECD, 2016; US EPA, 2011a; US EPA, 2011b; US EPA, 2011c). Accordingly, regulatory agencies, including the US EPA and EFSA, have accepted pursuing *in vitro* methods to replace traditional animal studies (US EPA, 2016; Fritsche et al., 2015).

Various cell lines originating from different segments of the gastrointestinal (GI) tract are commonly used in chemical risk assessment. Among the variety that are available, intestinal Caco-2 cells have been the most commonly used (Christensen et al., 2012) (APPENDICES X, XI, XII and XVIII).

3.2 NOEL CALCULATION:

NOEL Determination Using Cytotoxicity Testing:

The toxicity tests were performed using human, monkey and mice cell lines in addition to boar sperm with conversion for relevance in humans.

The three cell lines that were used for cytotoxicity studies of *Bacillus subtilis* DE111 included:

ATCC® CCL-1 L-929 cells, subcutaneous connective tissue; areolar and adipose tissue cells. The L929 cell line is an established substrate and has been commonly used for cytotoxicity evaluation (Poskus et al, 2009) (APPENDIX X).

Vero ATTC® CCL-81 cell lines from kidney tissue. This cell line is often utilized to represent normal healthy cells in toxicity studies (Hamdan et al., 2017) (APPENDIX XI).

Caco-2 ATCC® HTB-37 cell line from human epithelial colorectal adenocarcinoma. These cells were included as a representative model of the intestinal barrier (Sambuy et al., 2005) (APPENDIX XVIII).

Additionally, a boar sperm motility test was used to assess toxicity. Porcine spermatozoa are primary mammalian cells closely similar to human cells. The boar sperm motility assay is a suitable model for capturing multiple modes of action of drugs and other chemicals acting via mitochondrial disturbance as ejaculated spermatozoa are highly dependent on mitochondrial production and consumption of ATP for their metabolism which includes motility display (Vicente-Carrillo et al, 2015) (APPENDIX XII).

Determination of NOEL:

Using the four independent cell lines for toxicity studies, the amount of bacterial to human cell interaction *in vitro* was determined to be 3.2×10^8 bacterial cells to 2.0×10^5 human cells or a ratio of 1600 bacterial cells per human cell. Since there are over 1.67×10^{10} epithelial cells in the stomach and small intestine (Bianconi et al., 2013), the *in vitro* to *in vivo* conversion is achieved by multiplying the number of bacterial cells (1600) times the number of epithelial cells in the human body (1.67×10^{10}) which makes the NOEL equal 2.67×10^{13} CFU.

3.3 Calculation of Allowable Daily Intake (ADI)

Determination ADI using cell line testing and boar sperm:

Using the NOEL calculation from cytotoxicity studies and based on the fact that *Bacillus subtilis* has been consumed in food for decades, a reasonable and conservative safety factor or margin of safety (MOS) of 100 was used for adults and 1000 for children. The margin of safety is additionally augmented since it includes the conservative method for assessment of *in vivo* cell line cytotoxicity using concentrated *Bacillus subtilis* DE111, which is at least 100-fold more concentrated than would be the case for human cells in the stomach and 10-fold more concentrated than human cells in the small intestine.

The allowable daily intake (ADI) for adults and children was therefore calculated using the following equation:

ADI = NOEL/Safety factor.

Adults

ADI = $2.67 \times 10^{13}/100$, which is equal to 2.67×10^{11} CFU/ day

Children

ADI = $2.67 \times 10^{13}/1000$, which is equal to 2.67×10^{10} CFU / day

Determination of ADI Per Kilogram Body Weight

Furthermore, to define ADI in terms of CFU per body weight per day, the following calculation was used:

Bacillus subtilis DE111 ADI in CFU/average body weight/day

ADI = 2.67 X 10^{11} CFU/ body weight

Adult

The average body weight of an adult: 60kg

ADI = 2.67 X 10^{11} CFU/ 60 kg

ADI = 4.45 X 10^{9} CFU/ kg bw / day for adults or 1 g/kg bw/day

Children

Additionally, for the most conservative determination of allowable daily intake for a child, the youngest age (2-year-old) at the lowest weight (1st percentile) based on World Health Organization child growth standards was utilized: 8.7 kg

ADI = 2.67 X 10^{10} CFU/ 8.7 kg

ADI = 3.07 X 10^{9} CFU / kg bw / day for children or 500 mg/kg bw/day

Determination ADI cfu/kg bw/day using human clinical trials:

Two Clinical trials are at a serving of 1 Billion:

1.667 X 10^{7} CFU/kg bw/day or 2.5 ug/kg bw/day or 1.0 X 10^{9} CFU for 60 kg human

and two other trials are at a serving of 5 Billion:

8.335 X 10^{7} CFU/kg bw/day or 12.5 ug/kg bw/day or 5.0 X 10^{9} CFU for 60 kg human

Note: While consumption of any product would be diluted in the stomach and intestine by a dilution factor of at least 100 (Lien et al., 2016); the calculations above assumed no such dilution, so that safety could be assessed at the most concentrated level under the harshest of conditions.

Determination of ADI for Infants

For infants, an additional margin of safety of 10 was added to the ADI for children to obtain allowable daily intake for infants. This would equal 2.67 X 10^{9} CFU/day and 6.68 X 10^{8}CFU/ kg bw/day for 4 kg infant.

3.4 Estimated Daily Intake (EDI) and Dietary Exposure

Estimated Daily Intake - Adults

Deerland Probiotics and Enzymes has proposed the use of the *Bacillus subtilis* DE111 in products including baked goods and baking mixes; alcoholic beverages; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fats and oils; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy and cough drops; herbs, seeds,

spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups.

It is to be added to conventional foods at levels sufficient for adults to ensure at least 1 X 10^6 CFU/serving and no more than 1 X 10^{10} CFU/serving throughout the shelf-life of the product for an adult. The function of *Bacillus subtilis* DE111 is to serve as a probiotic microorganism. *Bacillus subtilis* DE111 will not proliferate in the foods and beverages to which it is added. The average individual consumes only about 20 servings/day of all food combined (Millen et al., 2006). It should be noted however, that based on the dietary guidelines for Americans, standard servings for children ages 2 to 6 years, women, and some older adults is 16 servings/day; for older children, teen girls, active women and most men, standard number is 21 servings/day; and for teen boys and active men, is 26 servings/day (US Department of Health and Human Services, 2015). Based on this information, if a person were to consume half of their 26 servings of food products containing *Bacillus subtilis* DE111/ day, their estimated daily intake would be 1.3 X 10^{11} CFU/day, less than the ADI of 4.45 X 10^9 CFU / kg bw /day, and less than 2.67 X 10^{11} CFU for a 60 kg individual.

Estimated Daily Intake - Children

The intended use level of *Bacillus subtilis* DE111 for a child is 1 X 10^9 CFU *Bacillus subtilis* DE111/serving or 1.15 X 10^8 CFU/kg bw/day, based on the 1st percentile weight (lowest) for a 2-year-old child. Assuming a conservative high-end consumption of 18.2 servings/day, the estimated daily intake (EDI) for children would equal 2.10 X 10^9 CFU/kg bw/day and 1.82 X 10^{10} CFU/child/day. The ADI is greater at 3.07 X 10^9 CFU/kg bw /day and 2.67 X 10^{10} CFU/child/day.

Estimated Daily Intake - Infants

Bacillus subtilis DE111 is intended to be added to non-exempt term infant formula (including milk-based, soy-based, modified, hydrolyzed, and amino acid-based formula powders and liquids) at levels up to 2 X 10^8 CFU per 100 mL of infant formula as ready for consumption.

Among healthy, full-term, formula-fed infants, highest energy consumption on a kcal/kg bw basis occurs in males 14-27 days old, who consume 121 and 143 kcals/kg bw/day at the 50th and 90th percentiles, respectively (Fomon, 1974). In female infants, the highest energy consumption at the 50th percentile occurs in the same age group (14-27 days: 117 kcal/kg bw/day) while the highest consumption at the 90th percentile (143 kcal/kg bw/day) occurs in the 8-13 days old group (note, this is identical to 90th percentile consumption in 14-27 days old males and only slightly higher than 90th percentile consumption in 14-27 days old females at 136 kcal/kg bw/day). Although dated, Fomon's data are consistent with more recent work based on the 2005-2012 National Health and Nutrition Examination Survey (NHANES) using the What We Eat in America food category classification system (Grimes et al., 2015) and the Feeding Infants and Toddlers study (FITS) 2008 (Butte et al., 2010). According to the NHANES analysis by Grimes et al., mean calorie intake by infants 0-5.9 months is 612.5±6.4 kcal/day while infants 6-11.9 months consume 847.3±13.3 kcal/day (Grimes et al., 2015). Based on the FITS 2008 analysis by Butte et al., mean and 90th percentile calorie intake was 611±6.9 and 779 kcal/day, respectively, in the 0-5 months age group and 854±11.3 and 1183 kcal/day, respectively, in the 6-11 months age group (Butte et al., 2010). Fomon's data provide finer graduations, reporting breaks down of calorie intake by gender on a mg/kg basis at the 10th, 50th and 90th percentiles from 8 days to 111 days old (approximately 3.5 months) divided into six age intervals

(8-13, 14-27, 28-41, 42-55, 56-83, and 84-111 days) (Fomon, 1974). That Fomon's data span only approximately 3.5 months versus data spanning 12 months provided by Grimes et al. and Butte et al. allows for a more conservative estimate as the percentage of calorie intake from infant formula declines with increasing age (Grimes et al., 2015).

Most cow-milk-based formulas for term infants (*i.e.*, "standard infant formulas) provide 67 kcal/100 mL (20 kcal/fl oz.) when ready for consumption, but formula concentrates may be mixed to yield higher calorie densities up to 101 kcal/100 mL (30kcal/fl oz.) (Martinez and Ballew, 2011). Based on an infant formula comparison chart provided by Martinez and Ballew, 67 kcal/100 mL (20 kcl/fl oz.) is the recommended target for cow milk- soy protein-, and amino acid-based, as well as modified and extensively hydrolyzed, formulas for healthy term infants without special medical needs (Martinez and Ballew, 2011). Using the most conservative estimates of 143 kcals/kg bw/day (the 90th percentile in girls 8-13 days and boys 14-27 days) and 67 kcal/100 mL formula as ready to consume and assuming formula accounts for 100% of energy consumption, approximately 213.4 mL/kg bw/day of infant formula would be consumed. At the maximum addition level of 2 X 10^8 CFU *Bacillus subtilis* DE111/100 mL of infant formula as ready for consumption a conservative high-end EDI is 4.27 X 10^8 CFU *Bacillus subtilis* DE111/kg bw. Considering the NOEL/ MOS 10,000, the ADI for infants would equal 2.67 X 10^9 CFU/infant/day and 6.68 X 10^8 CFU/ kg bw/day, which is greater than the EDI.

Only very young, exclusively formula-fed infants would be expected to consume 100% daily energy as infant formula. The percent of daily energy intake for formula declines with increasing age and introduction of solid food, with formula accounting for 65.4% and 47.1% of total daily energy intake in the 0-5.9- and 6-11.9-months groups, respectively (Grimes et al, 2015). Therefore, the true margin of safety for infants is expected to be even greater than the near ten-fold margin stated above.

4.0 SELF-LIMITING LEVELS OF USE

The amount offered 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 (no less than 1 mg and no more than 1 gram/dose). This level of use is consistent with dietary exposure and with the safety recognition by regulatory authorities in Japan, Europe, and Canada (EFSA 2007b; Gibson, 2015; NHPD, Health Canada, 2018).

5.0 EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Bacillus subtilis has been a component of food for decades in many countries by many cultures, and has been used in a variety of ways (Steinkraus, 2004). *Bacillus subtilis* Natto is made by fermenting cooked whole soybeans with a bacterial starter (*Bacillus subtilis*) (Tamang, et al, 2016). The finished product contains live viable *Bacillus subtilis*, which provides a health benefit for the consumer (Ping et al, 2016).

Bacillus subtilis, Natto, started in Japan, where it is usually served for breakfast. Because natto is made from soybeans that have not been dehulled, it is a whole, lightly processed, natural food (Tamang, 2015). *Bacillus subtilis* is also consumed in restaurants and homes across the United States (Shurtleff amd Aoyagi, 2012). Furthermore, *Bacillus subtilis* fermented foods are commonly found in several East Asian countries. There is *tan-shih* and *kan-shih* (salt-free soy nuggets) in China, *Joenkuk-jang* and *Damsue-jang* (both salted) in Korea, *thua-nao* in Thailand, *kinema* in Nepal (Tamang, 2015), and perhaps *sereh* in Bali

(Indonesia). Tanba, just north of Kyoto, has long been famous for its *Bacillus subtilis* (Shurtleff and Aoyagi, 2012).

As a common component of food, *Bacillus subtilis* is not restricted to any specific age groups. It is consumed among a wide age range.

6.0 NARRATIVE

6.1 *BACILLUS* BACTERIA UBIQUITOUS IN NATURE AND CONSUMED AS FOOD FOR HUMANS

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 may reach 10^7 CFU/g (Benno & Mitsuoka, 1986). A 2009 study by Hong *et al.* compared the density of *Bacillus subtilis* spores found in soil (about 10^6 spores per gram) to that found in human feces (about 10^4 spores per gram). The number of spores found in the human gut was too high to be attributed solely to consumption of the spores through food contamination and strongly suggested that *Bacillus subtilis* is a natural gut inhabitant.

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 & Fung, 1996). *Bacillus* species are the major microflora in soy beans and are responsible for their fermentation into soy food products and condiments (Ray *et al.* 2000; Inatsu *et al.* 2006). In Japan and in other countries, a culture of *Bacillus subtilis* subsp. *natto* is used to produce Natto, a popular traditional food made by fermenting cooked soy beans (Katz & Demain, 1977). In Japan, the food is most popular in the eastern regions, including Kantō, Tōhoku, and Hokkaido. (Shurtleff, & Aoyagi, 2012). *Bacillus subtilis* is occasionally used in other foods, such as natto sushi, natto toast, in miso soup, tamagoyaki, salad, as an ingredient in okonomiyaki, or even with spaghetti.

Natto made with *Bacillus subtilis* has a different nutritional makeup from raw soy beans, it includes the benefits of nutritious soy and softer dietary fiber without the high sodium content present in many other soy products, notably in miso. It 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), Many countries produce similar traditional soybean foods fermented with *Bacillus subtilis*, such as *shuǐdòuchǐ* of China, *cheonggukjang* of Korea, *thuanao* (ถั่วเน่า) 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.

Furthermore, *Bacillus* can also be found in wheat, grain, and whole-meal products with counts 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). Additionally, *Bacillus* are often present in raw milk, remain after pasteurization, and can be the predominant microflora in pasteurized milk products (Pendurkar & Kulkami, 1990).

In conclusion, humans are constantly exposed to the *Bacillus* species from the environment and foods with no evidence of infectious outbreaks due to these bacteria (except *Bacillus anthracis* and *Bacillus cereus*) or apparent ill effects. Some cases of infection associated with "non-pathogenic" *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 *Bifidobacteria* (Borriello *et al.* 2003). Therefore, *Bacillus subtilis* can be recognized as safe for human consumption.

6.2 General Safety of *Bacillus subtilis* for Infants

It is reasonable to add probiotic bacteria DE111 *Bacillus subtilis* to infant formula, since *Bacillus subtilis* and other probiotic bacteria have been isolated and characterized in human breast milk (Bhatt et al, 2012). Breast milk is an important and often sole nutrient source for infants. In addition to satisfying basic nutritional needs, breast milk is a source of beneficial bacteria that support a healthy microflora in the infant. Breast milk samples have revealed that *Bacillus subtilis* is among the healthful probiotic bacteria naturally present in human breast milk. Furthermore, *Bacilli* have been identified in the infant stools after birth through one year of age in healthy term infants (Palmer et al, 2007).

6.3 General Safety of *Bacillus subtilis*

The safety of *Bacillus subtilis* has been well established. The bacteria are 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 the natural tolerance for *Bacillus subtilis* and reflect their general safety.

Safety concerns include the potential presence of *Bacillus cereus* endotoxin genes and virulence factors and antibiotic resistance.

The FDA recognizes that substances derived from *Bacillus subtilis* are GRAS and were of common use in food prior to January 1, 1958 (FDA, 2018). Nontoxigenic and nonpathogenic strains of *Bacillus subtilis* are widely available and have been safely used in a variety of food applications. This includes consumption of Japanese Natto, commonly consumed in Japan, and contains as many as 10^9 viable cells per gram. Consumption of these foods is believed to be associated with numerous health benefits, such as enhanced immunity, reduced bone loss in post-menopausal women, and anti-allergy effects (Ouoba et al., 2004; Ikeda et al., 2006; Shurtleff, 2012).

In addition to the lack of adverse effects of *Bacillus subtilis* for human consumption in humans worldwide, new literature searches were completed in order to investigate results of studies conducted specifically in infants and elderly. A total of four randomized blinded placebo-controlled clinical trials and one cohort study in which elderly and children were administered *Bacillus subtilis* were located. None of the trials reported any treatment-related adverse effects. These trials are summarized with respect to safety aspects below.

A total of 650 mother-baby cohort were evaluated for intake of various foods including *Bacillus subtilis*, and their role in the development of eczema. Results showed that babies in the group with mothers who had lower intakes of *Bacillus subtilis* had significant incidence of eczema compared to those whose mothers had larger intake of the probiotic. More importantly, incidence of the eczema in the group taking *Bacillus subtilis* everyday was 6.7% compared to the group taking it two to three times a week or less, incidence 18.7%, $p = 0.020$ (Naoko et al, 2014).

No adverse effects were observed during another trial seeking to study the effect of a long-term *Bacillus subtilis* spores' oral treatment in children suffering from recurrent infectious diseases of the respiratory tract. Fifty-three children 5-9 years old were studied. Preliminary immunological laboratory evaluation demonstrated a complete return to the normal lymphomonocyte status after at least 2 months of treatment with *Bacillus subtilis* spores (Novelli et al, 1984).

In a Russian trial by O. M. and Yu to evaluate the effectiveness of *Bacillus subtilis* in the comprehensive therapy of children with gastrointestinal symptoms of food allergy, 34 children aged 3.5 to 12 years with food allergies with gastrointestinal complaints were enrolled. The main group included 18 children, the comparison group consisted of 16 patients. Children of the main group in addition to basic therapy received probiotic on the basis of *Bacillus subtilis* (1 dose 2 times a day for 20 days). The effectiveness of therapy was assessed by the rate and completeness of reduction of gastrointestinal symptoms and manifestations of comorbid allergic pathology. After taking the probiotic based on *Bacillus subtilis,* there were no complications or side effects in all patients, while episodes of abdominal pain and stool disorders were observed in children of the comparison group. (O.M & Yu, 2017).

The use of *Bacillus subtilis* for beneficial effects on immune health in elderly subjects was investigated. A dose of 2×10^9 spores per day of *Bacillus subtilis* was administered for a total 40 days to healthy elderly subjects (4 consumption periods of 10 days separated by 18-day washouts). 100 elderly subjects (60–74 years of age) were divided into placebo (39 females, 11 males; $n = 50$) and probiotic (40 females, 10 males; $n = 50$) groups. There were no abnormal values of biological parameters at the end of the study, and no clinically significant variation was observed during the study, on renal and hepatic functions. (Lafevre et al, 2015).

Another randomized, double-blinded, placebo-controlled clinical trial investigated the use of *Bacillus subtilis* therapy during antibiotic consumption as well the occurrence of *Clostridium difficile*-associated diarrhea (CDAD), abdominal symptoms, adverse effects and the acceptability of the probiotics in elderly patients (aged >56). Subjects were randomized into three groups: (i) probiotic group one, who received a probiotic, containing strains *Bacillus subtilis* and *Bacillus licheniformis* (2×10^9 colony forming units (CFU) per vial); (ii) probiotic group two, who received a probiotic containing *Bacillus subtilis* (2×10^9 CFU. per vial); and (iii) a placebo group, who received an inert composition in vials, formulated to be indistinguishable from the vials with probiotics. Treatment with *Bacillus* probiotics during antibiotic therapy significantly decreased the incidence of antibiotic-associated diarrhea (AAD) and adverse effects related to the use of antibiotics. Furthermore, *Bacillus subtilis* was well tolerated by the patients without side effects (Horosheva et al, 2014).

Bacillus subtilis has been proven to be of safe consumption in prenatal women, infants, and elderly with no adverse effects even after long-term consumption.

6.4 SAFETY OF *BACILLUS SUBTILIS* DE111

The safety of *Bacillus subtilis* DE111 based on its identification as *Bacillus subtilis* and that it is genetically 99.6% identical to Natto, a food consumed by humans for centuries in Asian and other cultures. Deerland Probiotics and Enzymes has shown, through 16S RNA, gyrB and full genome sequencing, that *Bacillus subtilis* DE111 is the same (95% or greater genome match) as *Bacillus subtilis* species as 168, R0179, CU1 and natto.

Safety analyses have been performed proving that it presents no hemolytic activity, plasmids, antibiotic resistance, detrimental effects on boar sperm motility or toxicity on human cell lines including CaCo2 and Vero Cells. *Bacillus subtilis* DE111 has never had an adverse event reported in any animal, clinical trial, or other human experience. Using a combination of 16S rRNA, *gyrB* and *gyrA* nucleotide analyses, the species was identified as *Bacillus subtilis*. Further characterization of the organism at the strain level was achieved using random amplified polymorphic DNA polymerase chain reaction (RAPD PCR) and pulsed field gel electrophoresis (PFGE) analyses. *Bacillus subtilis* did not demonstrate antibiotic resistance greater than existing regulatory cutoffs against clinically important antibiotics, did not induce hemolysis or produce surfactant factors, and was absent of toxigenic activity *in vitro*. (Pinchuk et al., 2002)

Gene mapping of the organism showed it is not closely related to *Bacillus cereus* or any other pathogenic strain. Furthermore, this *Bacillus subtilis* DE111 strain showed no *Bacillus cereus*-like endotoxin activity (described in Section 2.5.2). In addition, allergen (Section 2.5.3) and antibiotic resistance screen testing (Section 2.5.4) showed no positive results.

6.5 REGULATORY RECOGNITION OF *BACILLUS SUBTILIS* PRODUCTS FOR HUMANS AND ANIMALS

A number of regulatory guidelines and scientific decision trees have been published to assist in determining if a microbial strain intended for use as a probiotic is safe for human consumption. The Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food has also published guidelines for the Evaluation of Probiotics in Food (FAO/WHO, 2002). Within these guidelines, safety considerations outline a number of recommended safety tests for probiotics *in vitro* (Pariza et al. 2015). Furthermore, according to European Scientific Committee on Animal Nutrition, *Bacillus subtilis* was tested and showed no evidence of toxicity. Acute and chronic toxicity studies in animals also indicated safety of these strains. Likewise, *Bacillus subtilis* derived products are generally recognized as safe by the Food and Drug Administration (FDA), meaning they are not harmful to humans. (Olmos et al., 2014).

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 – self-affirmed GRAS in February 2012, by "Institut Rosell-Lallemand" for application as a heat-stable probiotic in baked goods, juices, and drinks.
- *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)
- *Bacillus subtilis* subsp. *inaquosorum* recognized by Japan's Ministry Health, Labor, and Welfare www.mhlw.go.jp/shingi/2008/09/dl/s0924-15n_0001.pdf - 20k - 2008-12-03
- 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)
- *Bacillus subtilis* PB6 granted Qualified Presumption of Safety (QSP) by EFSA as a feed additive for animals (porcine and avian), April 2012 (EFSA Journal 2012;10(5):2671 [8 pp.]. doi:10.2903/j.efsa.2012.2671)
- *Bacillus subtilis* DE111 granted "non-traditional food" status and not a novel food by the Australian Advisory Committee on Novel Foods (ACNF); the Committee deemed it not necessary to perform further public health and safety assessment following their hazard identification process.
- *Bacillus subtilis* has been used as the production organism for enzymes in nine GRAS Notifications to date; all received letters of no-objection from FDA.

In addition, the European Patent Office (EPO) has granted a patent for a new *Bacillus subtilis* subsp. *natto* strain to produce natto (EP2757150A1). The species has a 99.9% homologous rate to *Bacillus subtilis* subsp. *inaquosorum.*

6.6 REGULATORY RECOGNITION OF *BACILLUS SUBTILIS* AS QPS IN EUROPE

The European Food Safety Authority (EFSA) is required to assess the safety of biological agents which are regulated products that require authorization before marketing. In 2007, EFSA adopted the Qualified Presumption of Safety (QPS) approach for evaluating the safety of biological agents (EFSA, 2007). The aim of QPS is to evaluate safety using a harmonized risk assessment process and EFSA publishes a list of substances deemed qualified as safe in their published QPS list.

In 2007, EFSA granted *Bacillus subtilis* species Qualified Presumption of Safety (QPS) status, provided that no toxigenic activity was proven for the specific species (EFSA, 2007). This opinion was upheld in 2008 in a safety review of probiotic strains (EFSA, 2008) (Appendix IX). Moreover, *Bacillus subtilis* was identified at the strain and species level showed absence of transferable antimicrobial resistances (EFSA, 2012) and lacked toxigenic activity, in order to satisfy and support QPS status.

6.7 REGULATORY RECOGNITION OF *BACILLUS SUBTILIS* DE111 IN CANADA

Bacillus subtilis DE111 has obtained regulatory recognitions from Health Canada.

- *Bacillus subtilis DE111* – deemed by Health Canada as not novel and phylogenetically equivalent to *Bacillus subtilis* strain R0179, also not novel.
- *Bacillus subtilis* DE111 received Natural Product Number (NPN 80077102) per Canada's 2004-effective Natural Health Products Regulations which requires all-natural health products to have a product license and an NPN in order to be sold in Canada.

6.8 REGULATORY RECOGNITION OF *BACILLUS SUBTILIS* DE111 IN AUSTRALIA AND NEW ZEALAND

Bacillus subtilis DE111 has obtained regulatory recognitions from Food Standards Australia and New Zealand.

- According to Australia, New Zealand FSANZ, *Bacillus subtilis* DE111 meets the definition of 'non-traditional food' and 'novel food' in the Australia New Zealand Food Standards Code therefore, it does not require public health and safety assessment.

7.0 LIST OF SUPPORTING DATA AND INFORMATION

7.1 TOXICITY STUDIES

Pathogenic potential of some *Bacillus* strains is known; therefore, 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 - *Bacillus subtilis* and *Bacillus licheniformis* (Sorokulova *et al.* 2008), *Bacillus subtilis* and *Bacillus 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. *Bacillus subtilis* was safe *in vitro* toxicity studies and in chronic oral toxicity challenges, performed in rats (Tompkins *et al.* 2008). 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 the aforementioned *Bacillus* strains (Sorokulova *et al.* 2008) were in accordance with the safety records for *Lactobacillus* and *Bifidobacteria* (Sims, 1964).

7.1.1 SUB-ACUTE TOXICITY LITERATURE

Chronic toxicity has been studied on *Bacillus subtilis* with no signs of toxicity or histological changes in organs or tissues. 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 a 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.

Literature reviews showed that there have not been any sub-acute toxicity events with *Bacillus subtilis*.

7.1.2 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 in mice orally administered 5 X 10^7 to 2 X 10^{11} CFU/mouse. They found no ill effects in animals administered *Bacillus subtilis* IP or IV 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 hematological indexes measured in blood from treated or control animals. Tompkins *et al. (*2008) studied mice fed *Bacillus subtilis* 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.

7.1.3 CYTOTOXICITY TESTING OF *BACILLUS SUBTILIS* DE111

Cytotoxicity of *Bacillus subtilis* DE111 was determined *in vitro* with two cell lines, ATCC CCL-1 and CCL-81, by Nelson Laboratories (Salt Lake City UT, USA) and Emery Pharma Services (Emeryville CA, USA) respectively (Appendix X, Appendix XI). Minimal essential media (MEM) elution tests were executed to establish the cytotoxic effects and cellular destruction of *Bacillus subtilis* DE111 on Vero cell line monolayers. *Bacillus subtilis* DE111 did not exhibit cytotoxic effects on the ATCC CCL-1 or CCL-81 Vero cell lines *in vitro*. A lack of cell lysis and intracytoplasmic granules confirmed no discernable morphological cytotoxicity of *Bacillus subtilis* DE111.

Bacillus subtilis DE111 did not cause any detrimental effects on any of the tested cell lines. It can be regarded as an organism that does not cause cytotoxicity or toxic effects to cells.

7.1.4 TOXICITY TESTING OF *BACILLUS SUBTILIS* DE111

Toxicity of *Bacillus subtilis* DE111 was assessed using zones of hemolysis and boar sperm motility determination. When compared to *Bacillus cereus* cereulide-producing strain *Bacillus subtilis DE111* is a non-cereulide producing strain (Appendix XII).

Boar sperm motility determination is a suitable model to determine toxicity as sperm are highly dependent on mitochondrial production and can show toxicity through mitochondrial disturbance (Vicente-Carrillo et al, 2014). *Bacillus subtilis* DE111 has no impact on the motility of boar sperm compared to methanol and 30 ng Valinomycin (Appendix XII).

7.2 HUMAN CLINICALS WITH *BACILLUS SUBTILIS* DE111

7.2.1 THE EFFECT OF *BACILLUS SUBTILIS* DE111 ON THE DAILY BOWEL MOVEMENT PROFILE FOR PEOPLE WITH OCCASIONAL GASTROINTESTINAL IRREGULARITY

Objective

The objective of this study was to explore the safety and efficacy of *Bacillus subtilis* DE111 in a healthy population.

Methods
50 people were evaluated by their blood markers, stool profile, food diary and questionnaire while taking a probiotic *Bacillus subtilis* DE111 (1 billion CFU/day) or placebo over the course of 105-days. Safety of probiotic was measured using blood markers. The stools were scored based on the Bristol Stool Chart index during the 0, 45 and 105 days and safety was monitored throughout the study.

Results
No adverse events were noted. Statically significant results showed those in the *Bacillus subtilis* DE111 group moved to a healthier bowel index while those in the Placebo group stayed the same.

Conclusion
The study provided evidence that long-term consumption of *Bacillus subtilis* DE111 is safe for humans and may improve occasional constipation and/or diarrhea in healthy individuals (Appendix XIV).

7.2.2 Probiotic (*Bacillus Subtilis*) Supplementation During Offseason Resistance Training Improves Body Composition in Female Division I Athletes

Objective
The purpose of this investigation was to determine the safety and effects of probiotic *(Bacillus subtilis)* supplementation during offseason training in collegiate athletes.

Methods
Twenty-three, Division I female athletes 23 athletes participated in this study and were randomized into either a probiotic (n=11; *Bacillus subtilis* DE111) or placebo (n=12; PL) group. Athletes completed the same 10-week resistance training program during the offseason, which consisted of 3-4 workouts per week of upper and lower-body exercises and sport-specific training. Athletes consumed *Bacillus subtilis* DE111 (5 billion CFU/day) or PL supplement in conjunction with a recovery drink immediately following resistance and sport-specific training for the entire 10-week program. On weekend or non-training days, athletes consumed the supplement with a meal. Pre and post-training, all athletes underwent one-repetition maximum (1RM) strength testing (squat, deadlift, bench press), performance testing (vertical jump, pro-agility) and isometric mid-thigh pull testing (IMTP). Three compartment body composition estimation (BF%) was completed via BOD POD and BIA analysis, as well as muscle thickness (MT) measurement of the rectus femoris (RF) and vastus lateralis (VL) via ultrasonography. Separate repeated measures analyses of variance were used to analyze all data.

Results
No adverse effects were observed during this study. Significant ($p \leq 0.05$) main effects for time were observed for improved squat 1RM (*Bacillus subtilis* DE111: +15.2±6.9kg; PL: +17.7±4.9kg), deadlift 1RM (*Bacillus subtilis* DE111: +12.0±6.6kg; PL: +8.8±7.4kg), bench press 1RM (*Bacillus*

subtilis DE111: +4.3±4.5kg; PL: +3.5±3.1kg), vertical jump (*Bacillus subtilis* DE111: +1.0±0.6in; PL: +0.7±0.9in), RF MT (*Bacillus subtilis* DE111: +0.07±0.15cm; PL: +0.12±0.17cm) and BF%. Of these, a significant group x time interaction was noted for BF% (p=0.015), where greater reductions were observed in *Bacillus subtilis* DE111 (-2.05±1.38%) compared to PL (-0.2±1.6%). No other group differences were observed.

Conclusion
This data shows that probiotic *Bacillus subtilis* DE111 consumption in conjunction with post-workout nutrition is safe and may improve body composition in female Division I soccer and volleyball players following offseason training (Appendix XV).

7.2.3 Effects of Probiotic (*Bacillus subtilis* DE111) Supplementation on Immune Function, Hormonal Status, and Physical Performance in Division I Baseball Players

Objective
To determine the safety and effects of probiotic supplementation (*Bacillus subtilis* DE111; 1 billion CFU) on markers of immune and hormonal status in collegiate male athletes following 12 weeks of offseason training.

Methods
Twenty-five Division I male baseball athletes participated in this double blind, placebo-controlled, randomized study. Participants were randomly assigned to a probiotic (PRO; n = 13) or placebo (PL; n = 12) group. Pre- and post-training, all athletes provided resting blood and saliva samples. Circulating concentrations of testosterone, cortisol, TNF-α, IL-10, and zonulin were examined in the blood, while salivary immunoglobulin A (SIgA) and SIgM were assayed as indicators of mucosal immunity. Separate analyses of covariance (ANCOVA) were performed on all measures collected post intervention.

Results
No differences in measures of body composition or physical performance were seen between groups. TNF-α concentrations were significantly ($p = 0.024$) lower in PRO compared to PL, while there were no significant group differences in any other biochemical markers examined. A main effect for time was observed ($p < 0.05$) for increased testosterone ($p = 0.045$), IL-10 ($p = 0.048$),SIgA rate ($p = 0.031$), and SIgM rate ($p = 0.002$) following offseason training.

Conclusions
These data indicate that probiotic supplementation was safe, and had no effect on body composition, performance, hormonal status, or gut permeability. *Bacillus subtilis* DE111 attenuates circulating TNF-α in athletes (Appendix XVI).

7.2.4 Tolerance and Effect of a Probiotic *Bacillus subtilis* DE111 Supplement Delivered in Capsule Form

Objective

Probiotic supplements have shown benefit in increasing frequency and efficiency of bowel movements and some strains have shown to reduce serum glucose levels. *Bacillus subtilis* is used in fermentation of some foods for probiotic effects and may be useful in concentrated supplement form. The objectives of this clinical study were to determine if daily consumption of *Bacillus subtilis* strain DE111 at a dose of 5×10^9 CFU is safe for human consumption and effective at increasing frequency and improving consistency of bowel movements while increasing beneficial gut microbes and reducing pathogenic ones.

Methods
The tolerance and efficacy of encapsulated *Bacillus subtilis* DE111 was assessed in an average 20-day double blind, randomized, and placebo-controlled study.

Results
Most blood parameters remained within normal ranges throughout; however, fasted serum glucose levels in the probiotic group were significantly reduced. There was a significant increase in the average number of bowel movements per day within the placebo group (alpha ≤ 0.05; $P = 0.015$). Triglyceride levels maintained the same within the probiotic group, while the control group displayed a significant increase from pre to post based on a pair T-test (alpha ≤ 0.05; $P \leq 0.042$) (Figure 2). Additionally, significant differences in microbe colonization were present for *Bacillus subtilis* and *Bifidobacterium* in the fecal colony counts.

Conclusion
Daily consumption of *Bacillus subtilis* DE111 can be recognized as safe, and has potential to be effective as a supplement to improve glucose tolerance (Appendix XVII).

8.0 EXPERT PANEL STATEMENT

Determination of GRAS Status for *Bacillus subtilis* DE111

The undersigned, an independent panel of recognized experts (hereinafter referred to a "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 Probiotics and Enzymes to determine the GRAS status of *Bacillus subtilis* DE111 intended for use as a component in food. The scientific literature for safety and toxicity was made available to the Expert Panel. The Expert Panel independently evaluated these materials and others 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

Signature: (b) (6) Date: 10-30-18

Tiffany Weir, PhD
Associate Professor
Department of Food Science and Human Nutrition
Graduate Program in Cell and Molecular Biology
Colorado State University

Signature: (b) (6) Date: October 22, 2018

Martin L. Hudson, PhD
Associate Professor of Biology
Department of Molecular and Cellular Biology
Kennesaw State University

Signature (b) (6) Date: October 25, 2018

Mary Alice Smith, PhD

Professor (Retired)
Environmental Health Sciences Department
University of Georgia

Signature: (b) (6) Date : Oct 27, 2018

Joseph V. Rodricks, PhD, DABT
Principal, Ramboll

9.0 APPENDICES

9.1 APPENDIX I – REFERENCES

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9.2 APPENDIX II- SHELF STABILITY OF *BACILLUS SUBTILIS* DE111 FOR 24 MONTHS

Introduction

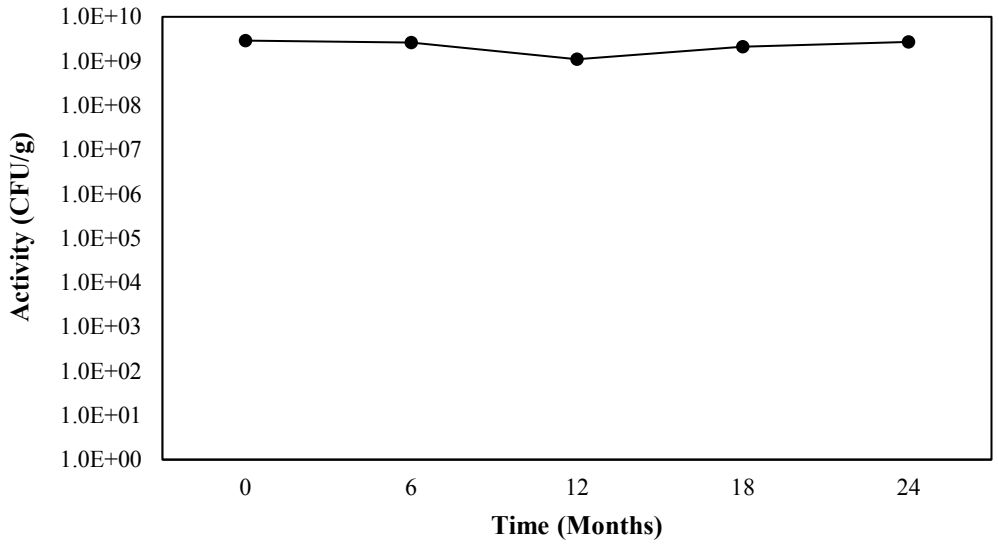
The official methods of analysis of AOAC international was used to determine targeted enumeration of aerobic probiotic bacteria containing samples, raw materials, in-process goods and finished goods for 24 months.

Materials and Methods

Bacillus subtilis DE111 samples were tested using the AOAC official methods of analysis, 990.12 and 966.23B method and 3M Petrifilm product instructions.

Results

Shelf stability of *Bacillus subtilis* DE111 for 24 months



Discussion

Bacillus subtilis DE111 was stable over 24 months under manufacturer recommended storage conditions. No loss was detected within the range of the assay.

9.3 APPENDIX III - SURVIVABILITY OF *BACILLUS SUBTILIS* DE111

Introduction

The objective of these experiment was to determine the survivability of *Bacillus subtilis* DE111 in acidic and bile salt conditions in nutrient broth media for 24 hours.

Materials and Methods

The United States Pharmacopeia (<2040> Disintegration and Dissolution of Dietary Supplements) was performed in order to establish the survivability of *Bacillus subtilis* DE111 under acidic conditions. Simulated gastric fluid was prepared, with and without pepsin, at a final pH of 1.2. *Bacillus subtilis* DE111 was inoculated in both gastric fluid preparations and incubated at 37 ± 1°C for 1 hour. Cultures were serially diluted, inoculated on 3M plates, and incubated at 37 ± 1°C for 24 hours.

Subsequently, the survivability of DE111 in acidic and bile salt conditions were assayed following the publication by Jiang et al. The method for tolerance to acid and bile salt concentration was done with some minor modifications. Overnight cultures of DE111 in nutrient broth (24 h) were inoculated in nutrient broths that were adjusted to pH 4.5, 3.5, and 2.5 with HCl (1.0 M) and in non-acidified broth (pH 6.9) which served as a control. To test for bile salt survivability, cultures were inoculated into nutrient broths supplemented with 0.15, 0.30, and 0.45% (wt/vol) of ox gall (Sigma-Aldrich Bile bovine, CAS no. 8008-63-7). All cultures were incubated at 37°C for 0, 3, 6, 12, and 24 hours. Following incubation, samples were serially diluted and plated in 3M Petrifilm aerobic count plates.

Results:

Bacillus subtilis DE111 viability under (USP 32/<2040>) acidic conditions after 24-hour incubation (Figure 1).

Figure 1. Survivability of DE111 under acidic (pH 1.2) conditions.

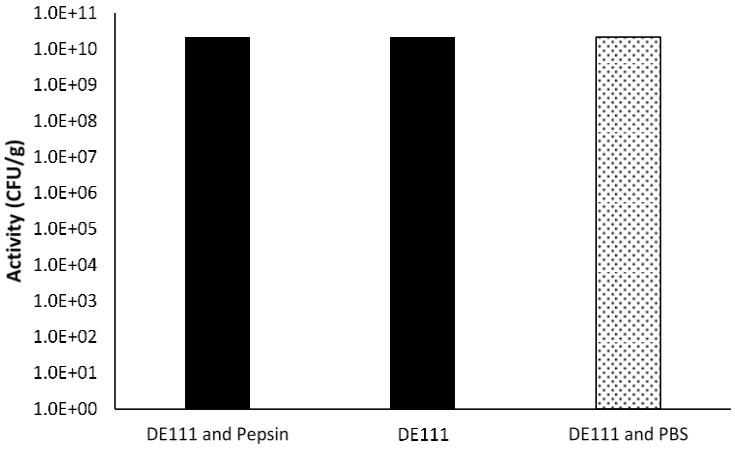
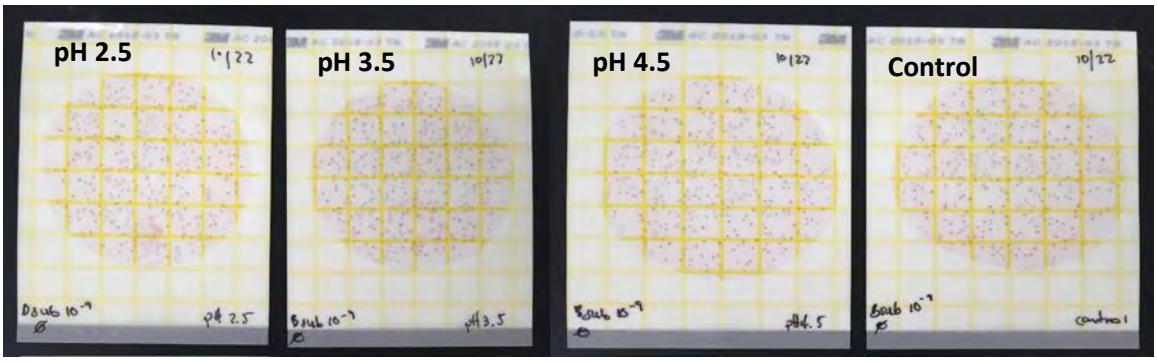


Figure 2. Survivability of DE111 under acidic (pH 1.2) conditions.

Acid Survivability



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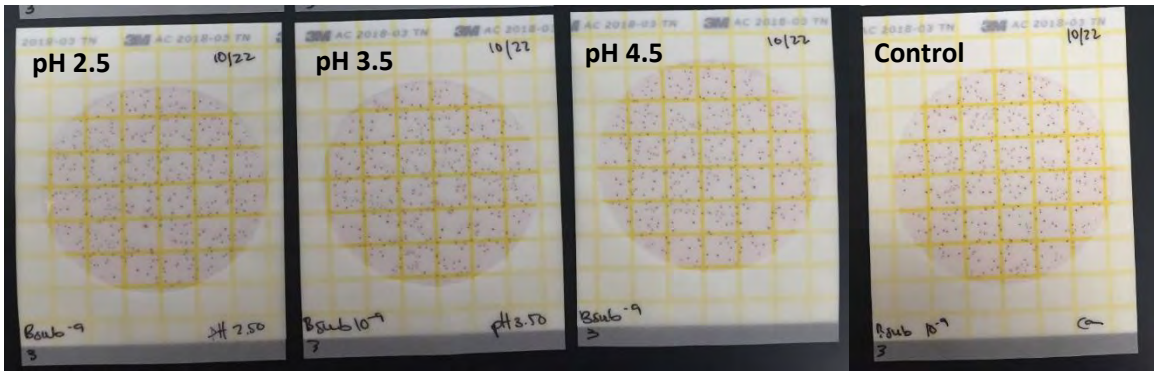
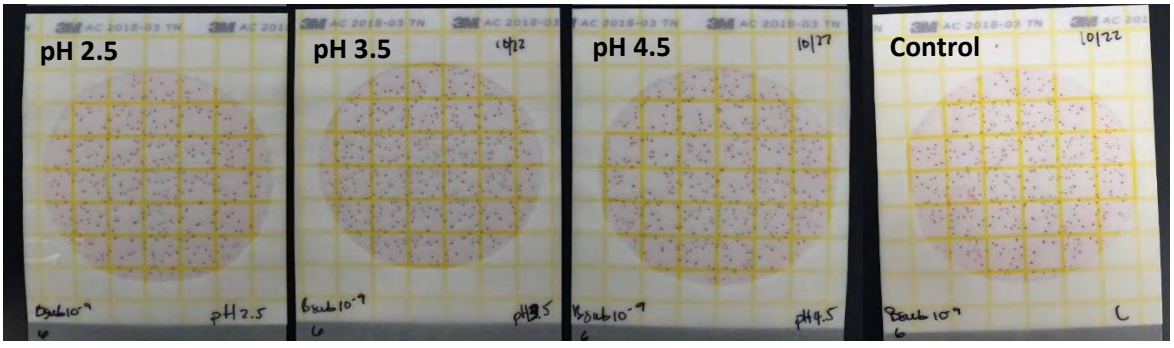


Time: 3 hours



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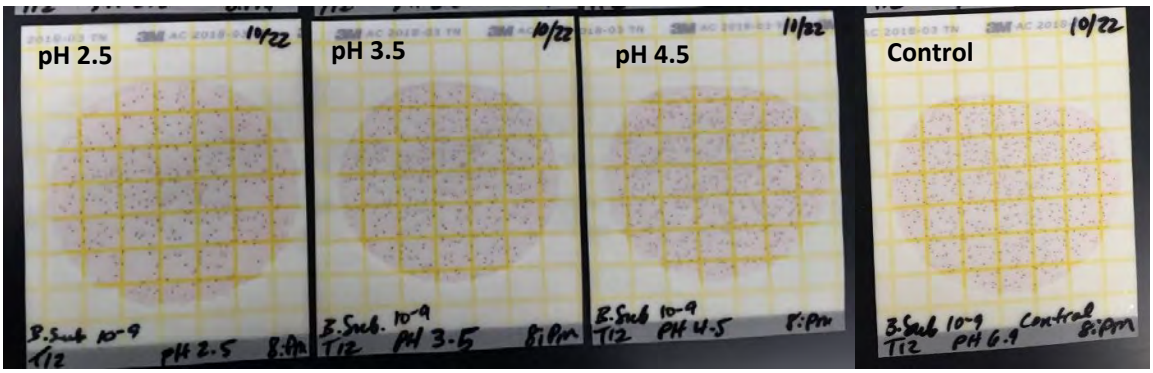
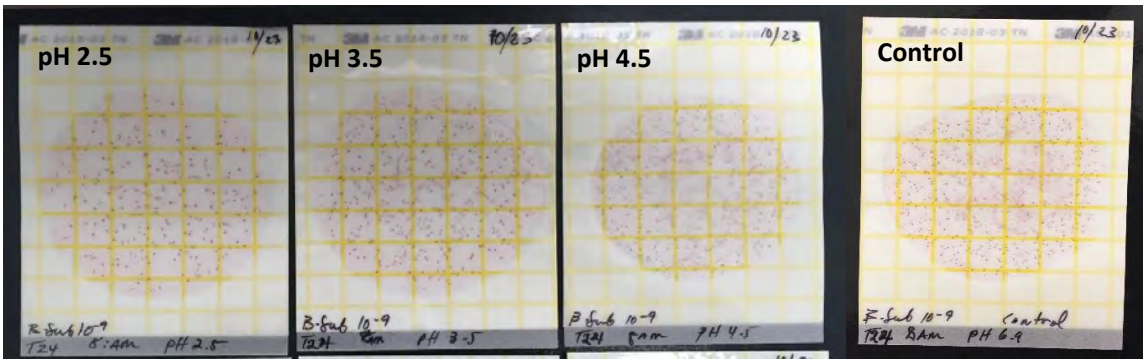
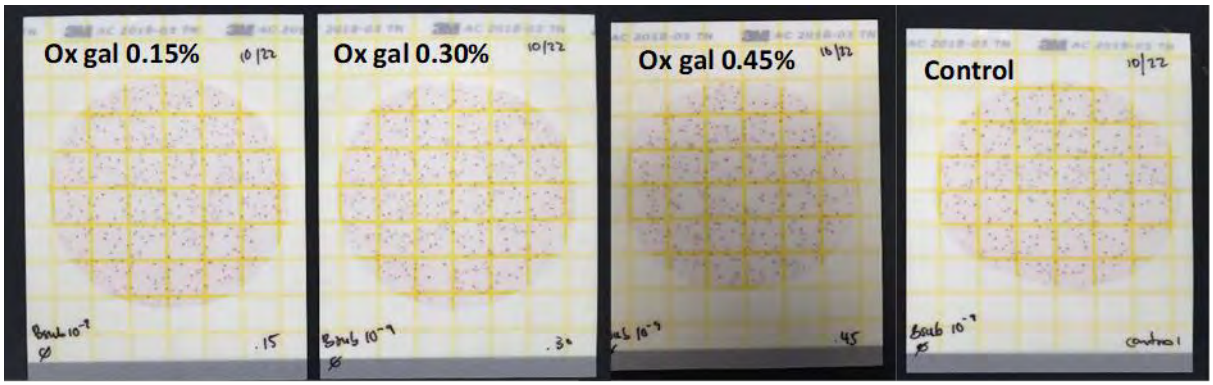


Time: 12 hours

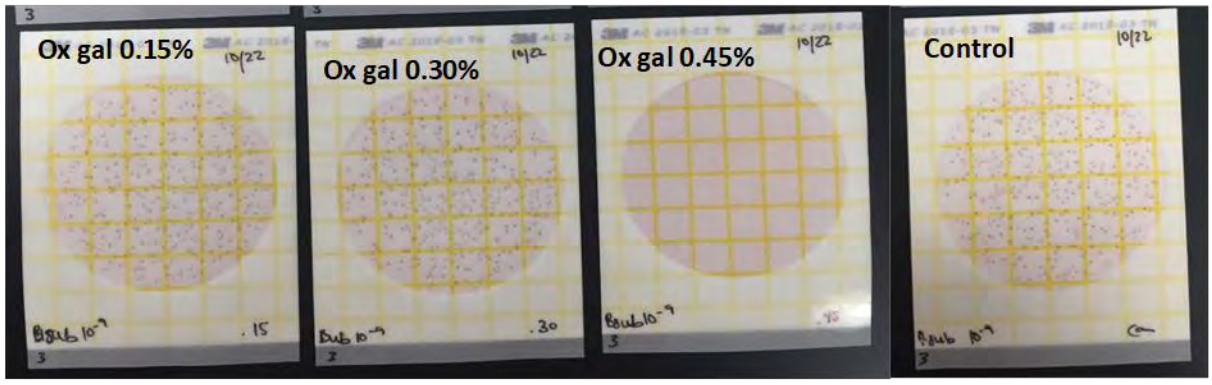


Time: 24 hours

Bile Salt Survivability



Time : 0 hours



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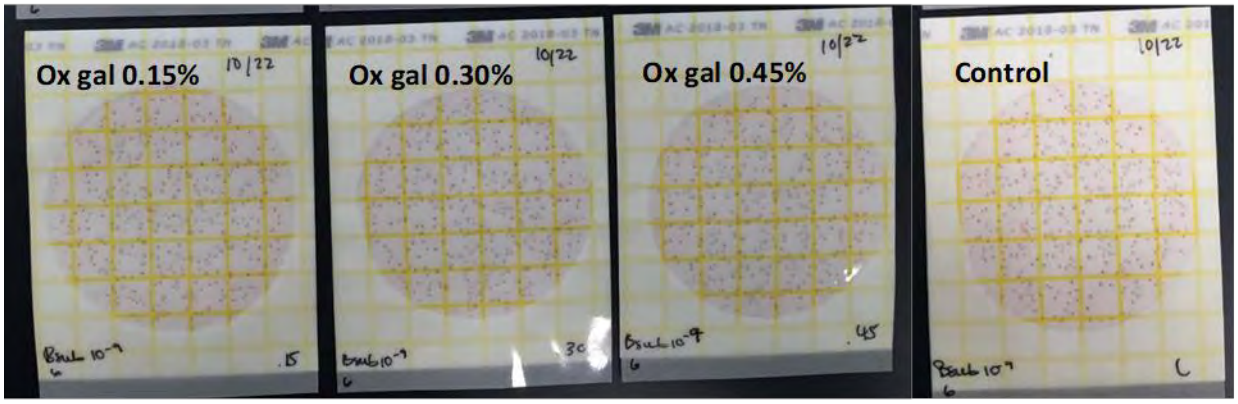

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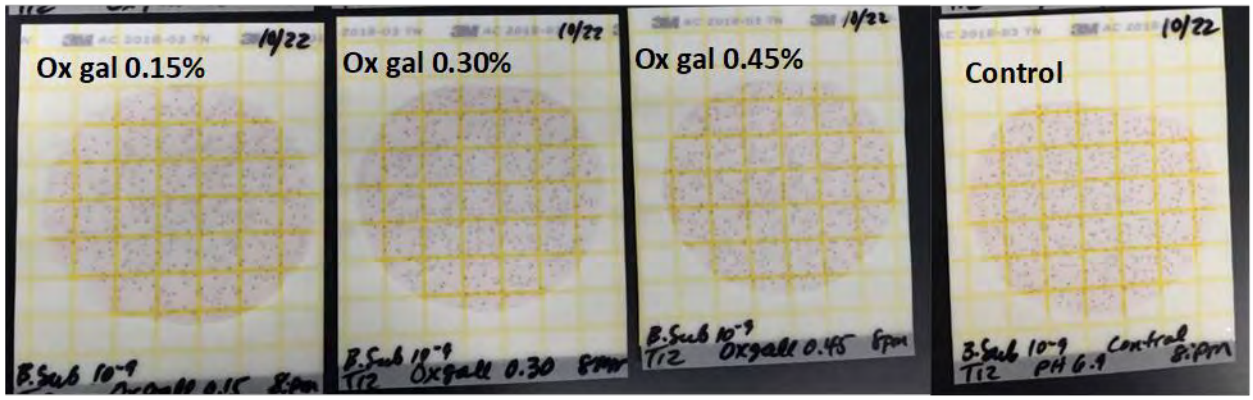

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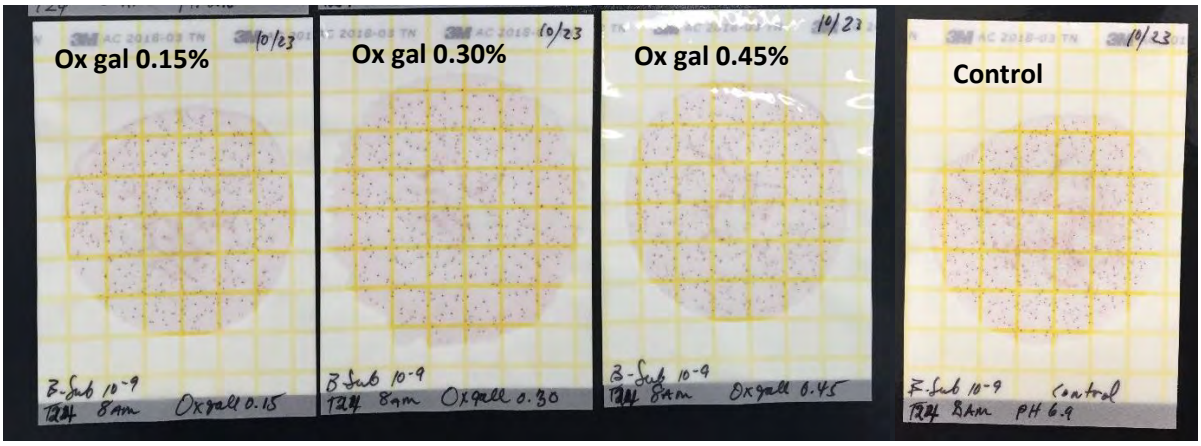


Time: 24 hours

Dilution	Average Acidified Nutrient Broth	Average Bile Salt Nutrient Broth
10^7	TNTC	TNTC
10^9	$40x10^{10}$ CFU/g	$40x10^{10}$ CFU/g

Conclusion:

Total DE111 counts did not reduce in viability / concentration after contact with acidic fluid (USP 32/<2040>) or acidic/salt concentrated nutrient broth for 24 hours. Based on these results it was determined that DE111 is not sensitive to acid or bile and it is capable of maintaining viability in low pH and high salt concentrations.

References:

Jiang M., Zhang, F., Wan, C., Xiong, Y., Shah, N. P., Wai, H., and Tao, X. (2016) Evaluation of probiotic properties of *Lactobacillus plantarum* WLPL04 isolated from human breast milk. *Journal of Dairy Science, Volume 99, Issue 3, Pages 1736-1746*. https://doi.org/10.3168/jds.2015-10434.

9.4 APPENDIX IV- 16S RDNA IDENTIFICATION AND GENOTYPIC IDENTIFICATION OF DE111

Charles River Microbial Solutions, Newark, DE, USA, Cornell University, Ithica, NY

charles river | microbial solutions

Processing Lab:
614 Interchange Blvd.
Newark, DE 19711
www.criver.com/accugenix
Del-CustomerSupport@crl.com
Phone: +1.302.292.8888

Accugenix®
AccuGENX-ID® Report
SOP-GEN-017

Customer:	Deerland Enzymes	Account: 601043	(DLZ1)
Address:	3800 Cobb International Blvd. NW, Kennesaw, GA, 30152, United States		
Accugenix C#:	C2806325-20171023018	ID Request Form #:	289784
Customer Sample ID:	DE111	Due Date:	2017-10-23

Accugenix Database Search Result - BacSeq

Identification: **Bacillus halotolerans / mojavensis / subtilis / tequilensis**

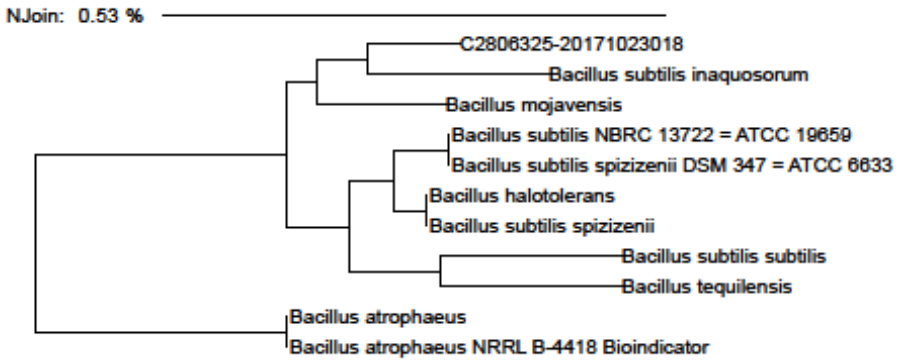
Confidence Level: **Species***

*The Unknown matches two or more closely related species that cannot be differentiated by 16S rDNA. We may be able to distinguish between the above species using ProSeq. Contact technical support to request this service.

Sequence Alignment

Alignment: 535 C2806325-20171023018
0.28 % 535 Bacillus mojavensis
0.28 % 535 Bacillus subtilis inaquosorum
0.28 % 535 Bacillus halotolerans
0.28 % 535 Bacillus subtilis spizizenii
0.37 % 535 Bacillus subtilis NBRC 13722 = ATCC 19659
0.37 % 535 Bacillus subtilis spizizenii DSM 347 = ATCC 6633
0.56 % 535 Bacillus subtilis subtilis
0.56 % 535 Bacillus tequilensis
0.75 % 535 Bacillus atrophaeus
0.75 % 535 Bacillus atrophaeus NRRL B-4418 Bioindicator

Neighbor Joining Tree



Not intended for in vitro diagnostic use

Page 1 of 1

Prepared By Robert Stevens at Newark, DE, United States on 2017-10-17 15:06:51
Reviewed By Kerry Falgowski at Newark, DE, United States on 2017-10-18 14:22:58
QA Approved By Cynthia Haskins at Newark, DE, United States on 2017-10-23 13:19:08
This document has been signed electronically by the individual(s) shown above, in compliance with FDA regulation CFR 21 Part 11.

Rev. 22May2017 EH

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www.criver.com/accugenix
Del-CustomerSupport@crl.com
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SOP-GEN-017

Customer:	Deerland Enzymes	Account: 601043	(DLZ1)
Address:	3800 Cobb International Blvd. NW, Kennesaw, GA, 30152, United States		
Accugenix C#:	C2806326-20171023018	ID Request Form #:	289784
Customer Sample ID:	R0179	Due Date:	2017-10-23

Accugenix Database Search Result - BacSeq

Identification: **Bacillus halotolerans / mojavensis / subtilis / tequilensis**

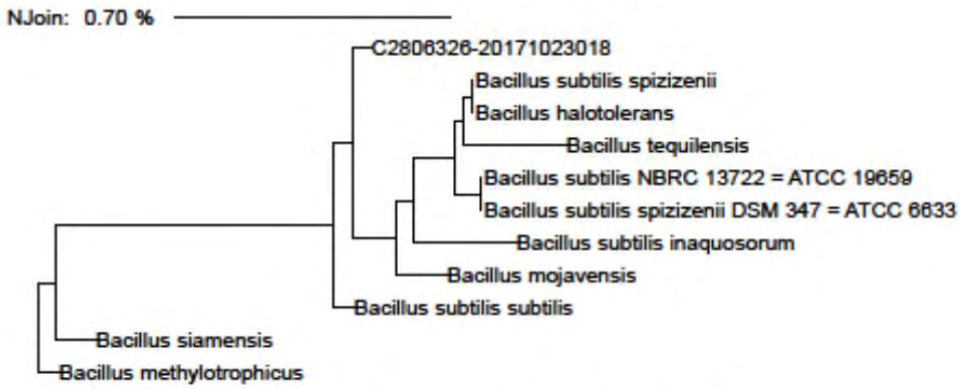
Confidence Level: **Species***

*The Unknown matches two or more closely related species that cannot be differentiated by 16S rDNA. We may be able to distinguish between the above species using ProSeq. Contact technical support to request this service.

Sequence Alignment

Alignment: 535 C2806326-20171023018
0.19 % 535 Bacillus subtilis subtilis
0.28 % 535 Bacillus mojavensis
0.37 % 535 Bacillus tequilensis
0.37 % 535 Bacillus subtilis NBRC 13722 = ATCC 19659
0.37 % 535 Bacillus subtilis spizizenii DSM 347 = ATCC 6633
0.47 % 535 Bacillus subtilis spizizenii
0.47 % 535 Bacillus subtilis inaquosorum
0.47 % 535 Bacillus halotolerans
0.84 % 535 Bacillus siamensis
0.84 % 535 Bacillus methylotrophicus

Neighbor Joining Tree



Prepared By Robert Stevens at Newark, DE, United States on 2017-10-17 15:06:51
Reviewed By Kerry Falgowski at Newark, DE, United States on 2017-10-18 14:22:58
QA Approved By Cynthia Haskins at Newark, DE, United States on 2017-10-23 13:19:08

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SOP-GEN-017

Customer:	Deerland Enzymes	Account: 601043	(DLZ1)
Address:	3800 Cobb International Blvd. NW, Kennesaw, GA, 30152, United States		
Accugenix C#:	C2806327-20171023018	ID Request Form #:	289784
Customer Sample ID:	168	Due Date:	2017-10-23

Accugenix Database Search Result - BacSeq

Identification: **Bacillus halotolerans / mojavensis / subtilis / tequilensis**

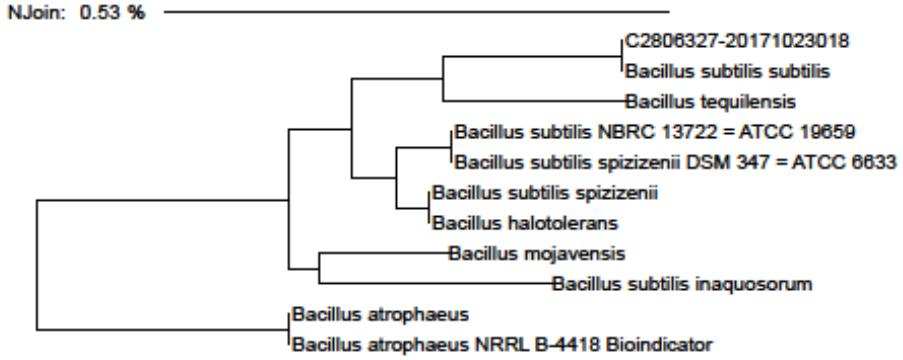
Confidence Level: **Species***

*The Unknown matches two or more closely related species that cannot be differentiated by 16S rDNA. We may be able to distinguish between the above species using ProSeq. Contact technical support to request this service.

Sequence Alignment

Alignment: 535 C2806327-20171023018
0.00 % 535 Bacillus subtilis subtilis
0.37 % 535 Bacillus tequilensis
0.37 % 535 Bacillus subtilis NBRC 13722 = ATCC 19659
0.37 % 535 Bacillus subtilis spizizenii DSM 347 = ATCC 6633
0.47 % 535 Bacillus subtilis spizizenii
0.47 % 535 Bacillus halotolerans
0.47 % 535 Bacillus mojavensis
0.65 % 535 Bacillus subtilis inaquosorum
0.75 % 535 Bacillus atrophaeus
0.75 % 535 Bacillus atrophaeus NRRL B-4418 Bioindicator

Neighbor Joining Tree



Not intended for in vitro diagnostic use

Prepared By Robert Stevens at Newark, DE, United States on 2017-10-17 15:06:51
Reviewed By Kerry Falgowski at Newark, DE, United States on 2017-10-18 14:22:58
QA Approved By Cynthia Haskins at Newark, DE, United States on 2017-10-23 13:19:08

Rev. 22May2017 EH

Genotypic Identification

Deerland Enzymes, Inc. corroborated with Cornell University (Ithica NY, USA) and Microbe Inotech Laboratories, Inc. (St. Louis MO, USA) for genome sequencing and identification. Cornell University used the following five reference genomes for comparative analysis: *Bacillus subtilis* subsp. *subtilis* str. 168, *Bacillus subtilis* subsp. *subtilis* str. BAB-1, *Bacillus subtilis* subsp. *natto*, *Bacillus subtilis* subsp. *inaquosorum*, and *Bacillus subtilis* subsp. *spizizenii*. Microbe Inotech used the following six reference genomes for comparative analysis: *Bacillus subtilis* subsp. *subtilis* str. 168, *Bacillus subtilis* subsp. *subtilis* str. BAB-1, *Bacillus subtilis* subsp. *natto* str. BEST195, *Bacillus subtilis* subsp. *inaquosorum* str. KCTC13429, *Bacillus subtilis* subsp. *spizizenii* str. W23, and *Bacillus subtilis* subsp. *spizizenii* str. TU-B-10. The genome sequence was provided to National Center for Biotechnology Information (NCBI) GenBank for inclusion in the genome database (*Bacillus subtilis* DE111 NCBI reference sequence: NZ_CP013984.1).

WGS DNA Composition

Cornell University and Microbe Inotech Laboratories, Inc.

The whole genome sequence was obtained for the *Bacillus subtilis* isolate, assembled, and annotated by Cornell University and Microbe Inotech Laboratoies, Inc. Bioinformatics analysis was completed at Cornell University and at Deerland Probiotics and Enzymes (Kennesaw GA, USA). DNA nucleotide content, base pair lengths for *Bacillus subtilis* DE111 genome, and marker sequences are shown below (**Table 1**).

Table 1. Nucleotide content, base pair length, and marker sequences of DE111.

	Genome	gyrB	16S rRNA	gyrA	*RimM* 16S
Length	4,143,890	1,968	1,352	2,421	525
GC Content	43.90%	45.58%	54.96%	44.40%	42.10%

Characters	**Genome**	**gyrB**	**16S rRNA**	**gyrA**	***RimM* 16S**
A	1,156,978 (27.9%)	618 (31.4%)	270 (20.0%)	773 (31.9%)	187 (35.6%)
C	912,845 (22.0%)	378 (19.2%)	422 (31.2%)	489 (20.2%)	84 (16.0%)
G	906,210 (21.9%)	519 (26.4%)	321 (23.7%)	586 (24.2%)	137 (26.1%)
T	1,167,857 (28.2%)	453 (23.0%)	339 (25.1%)	573 (23.7%)	117 (22.3%)
AA	402,657	233	56	281	77
AC	189,866	94	96	133	30
AG	228,940	155	68	163	40

AT	335,515	135	50	195	39
CA	275,666	120	92	164	27
CC	192,734	49	128	86	11
CG	212,038	120	95	132	32
CT	232,407	89	107	107	14
GA	269,646	180	63	214	56
GC	255,369	137	93	135	24
GG	189,643	119	78	130	31
GT	191,551	83	86	107	26
TA	209,009	85	59	114	26
TC	274,876	98	105	135	19
TG	275,588	125	80	161	34
TT	408,384	145	95	163	38

Note: length measured as bp for whole genome, gyrB gene, 16S rRNA, and gyrA gene. RimM 16S gene was used as an additional housekeeping marker sequence.

GyrB Gene *Deerland Enzymes, Inc.*

Gene sequence analysis using the gyrB gene polymorphism, a well-established method for species level discrimination of prokaryotes (**Bavykin et al., 2004; Wang et al., 2007**) showed that *Bacillus subtilis* DE111 was most related (99% identity) to the *Bacillus subtilis* subsp. *inaquosorum* group (**Table 2**) (**Rooney et al., 2009**).

Table 2. Distance matrix of *gyrB* gene.

***Bacillus subtilis* subsp.**	***subtilis* BAB-1**	***natto* BEST195**	***subtilis* 168**	**DE111**	***inaquosorum* KCTC 13429**	***spizizenii* W23**	***spizizenii* TU-B-10**
subtilis BAB-1	…	98.984	99.238	95.935	95.325	94.157	94.919
natto BEST195	98.984	…	99.339	95.681	95.071	93.902	94.665
subtilis 168	99.238	99.339	…	95.884	95.274	94.106	94.868
inaquosorum DE111	95.935	95.681	95.884	…	98.78	95.833	96.138
inaquosorum KCTC 13429	95.325	95.071	95.274	98.78	…	95.783	96.24
spizizenii W23	94.157	93.902	94.106	95.833	95.783	…	97.409
spizizenii TU-B-10	94.919	94.665	94.868	96.138	96.24	97.409	…

The representative genomes were previously reviewed, curated by NCBI, and coordinated with the UniProt Consortium (**NCBI, 2016; UniProt, 2016**). R package SequinR coupled with the UniProt Consortium analysis was used to compare whole genome sequences (WGS) and *GyrB* sequence of

Bacillus subtilis DE111 and six reference sequences (**Table 3 and 4**, **respectively**) for base pair length and GC content. Independent whole genome sequence (WGS) analysis by Microbe Inotech Laboratories identified DE111 with a homology most similar to *Bacillus subtilis* subsp. *inaquosorum* str. KCTC 13429.

Table 3. Whole genome sequence comparison.

***Bacillus subtilis* subsp.**	**Accession No.**	**% GC**	**Sequence Length**
inaquosorum DE111	NZ_CP013984	43.90%	4,143,890
inaquosorum KCTC 13429	NZ_AMXN01000003	43.70%	4,342,448
natto BEST195	NC_017196	43.50%	4,105,380
spizizenii W23	NC_014479	43.90%	4,027,676
spizizenii TU-B-10	NC_016047	43.80%	4,207,222
subtilis 168	NZ_CP010052	43.50%	4,215,619
subtilis BAB-1	NC_020832	43.90%	4,021,944

Table 4. *gyrB* gene sequence (1968bp) comparison.

***Bacillus subtilis* subsp.**	**Accession No.**	**% GC**
inaquosorum DE111	NZ_CP013984	45.60%
inaquosorum KCTC 13429	NZ_AMXN01000003	45.40%
natto BEST195	NC_017196	45.40%
spizizenii W23	NC_014479	44.70%
spizizenii TU-B-10	NC_016047	45.20%
subtilis 168	NZ_CP010052	45.80%
subtilis BAB-1	NC_020832	45.30%

***16S rRNA** Deerland Probiotics and Enzymes*

WGS and 16S rRNA analysis of *Bacillus subtilis* DE111, as compared to the six reference strains, exhibited an average nucleotide identity (ANI) score for 16S rRNA of 99.4% when compared to *Bacillus subtilis* subsp. *inaquosorum* str. KCTC 13429 (**Table 5**). The genome size (4.32 Mbp) and GC content (43.9%) for *Bacillus subtilis* DE111 was comparable to the six reference strains.

Table 5. 16S rRNA comparison (100% coverage, 525bp ALN length).

***Bacillus subtilis* subsp.**	**Accession No.**	**ANI**	**GC %**
inaquosorum DE111	NZ_CP013984.1	100	43.9

inaquosorum KCTC 13429	NZ_AMXN01000003	99.43	44.9
spizizenii TU-B-10	CP002905.1	94.86	43.8
spizizenii W23	CP002183.1	94.1	43.9
natto BEST195	AP011541.2	93.14	42.2
subtilis 168	NC_000964.3	93.14	43.5
subtilis BAB-1	NC_020832.1	92.95	43.9

Phylogenetic Placement *Deerland Probiotics and Enzymes, Inc.*

Genome-to-genome distance calculation (GGDC), a digital gold standard, is as reliable as DNA-DNA hybridization (DDH) (**Auch et al., 2010**). GGDC holds more discriminatory power for subspecies delineation and subsequently, was used as a confirmation of multiple alignment and phylogenetic analyses. GGDC yielded three calculation-based models that further verified *Bacillus subtilis* DE111 is a close relative to *Bacillus subtilis* subsp. *inaquosorum* str. KCTC 13429 and exhibited negligible similarities in homology when compared to *Bacillus cereus* ATCC 14579 (**Table 6**).

Table 6. Genome-to-genome distance calculation (GGDC) comparison

***Bacillus subtilis* subsp.**	DDH Model I	Model C.I.	Distance	DDH ≥ 70%	
inaquosorum KCTC 13429	91.6	[88.6 - 93.9%]	0.0717	97.82	0.21
subtilis BAB-1	86.2	[82.6 - 89.2%]	0.101	95.64	0.01
spizizenii W23	84.8	[81.1 - 87.9%]	0.1081	94.85	0.01
spizizenii TU-B-10	84.5	[80.8 - 87.7%]	0.1095	94.67	0.07
subtilis 168	81.6	[77.7 - 84.9%]	0.1245	92.49	0.38
natto BEST195	77.7	[73.7 - 81.2%]	0.1439	88.42	0.4
ATCC 14579* (NC_004722_WGS)	12.7	[10.1 - 16%]	0.9863	0	8.62

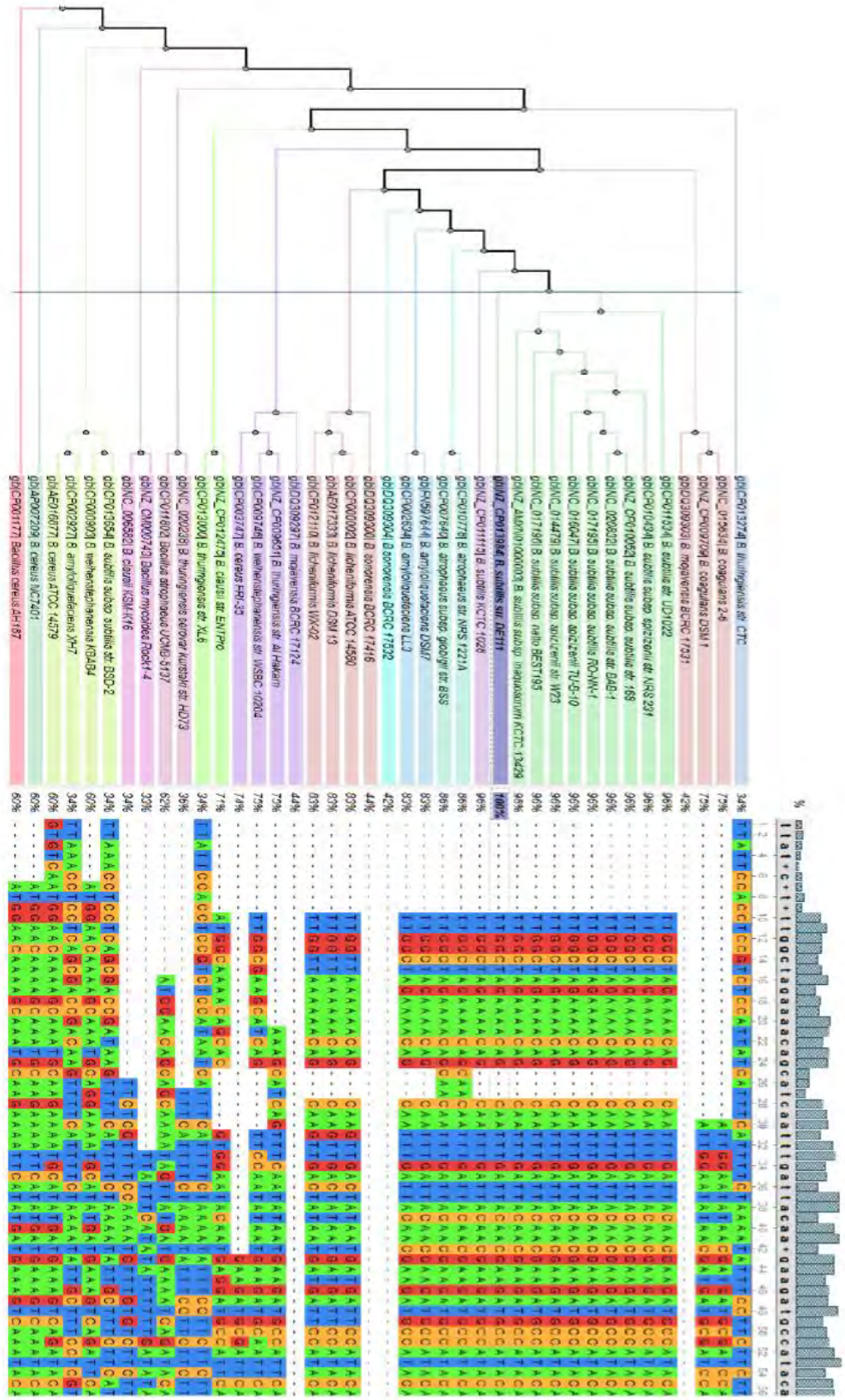
**Bacillus cereus* ATCC14579.

Although the conserved 16S rRNA sequence is a well-established method to compare and study phylogenies in bacteria, the high percentage of sequence similarity between closely related species limits its usefulness (**Wang et al., 2007**). High rates of 16S rRNA sequence similarity in closely related bacterial species are due to a slower rate of molecular evolution. Past research (**Bavykin et al., 2004; Wang et al., 2007**) supports the validity of using *gyrB* sequences as taxonomic biomarkers due to their rate of base substitutions and significant and reliable correlation with DNA-DNA Hybridization analysis (**Dauga et**

al., 2002; Kasai et al., 1998; Wang et al., 2007). The *gyrB* encodes DNA gyrase B, a type II topoisomerase that plays an important role in DNA replication. Gyrase A and gyrase B subunits are encoded by the *gyrA* gene and *gyrB* gene, respectively.

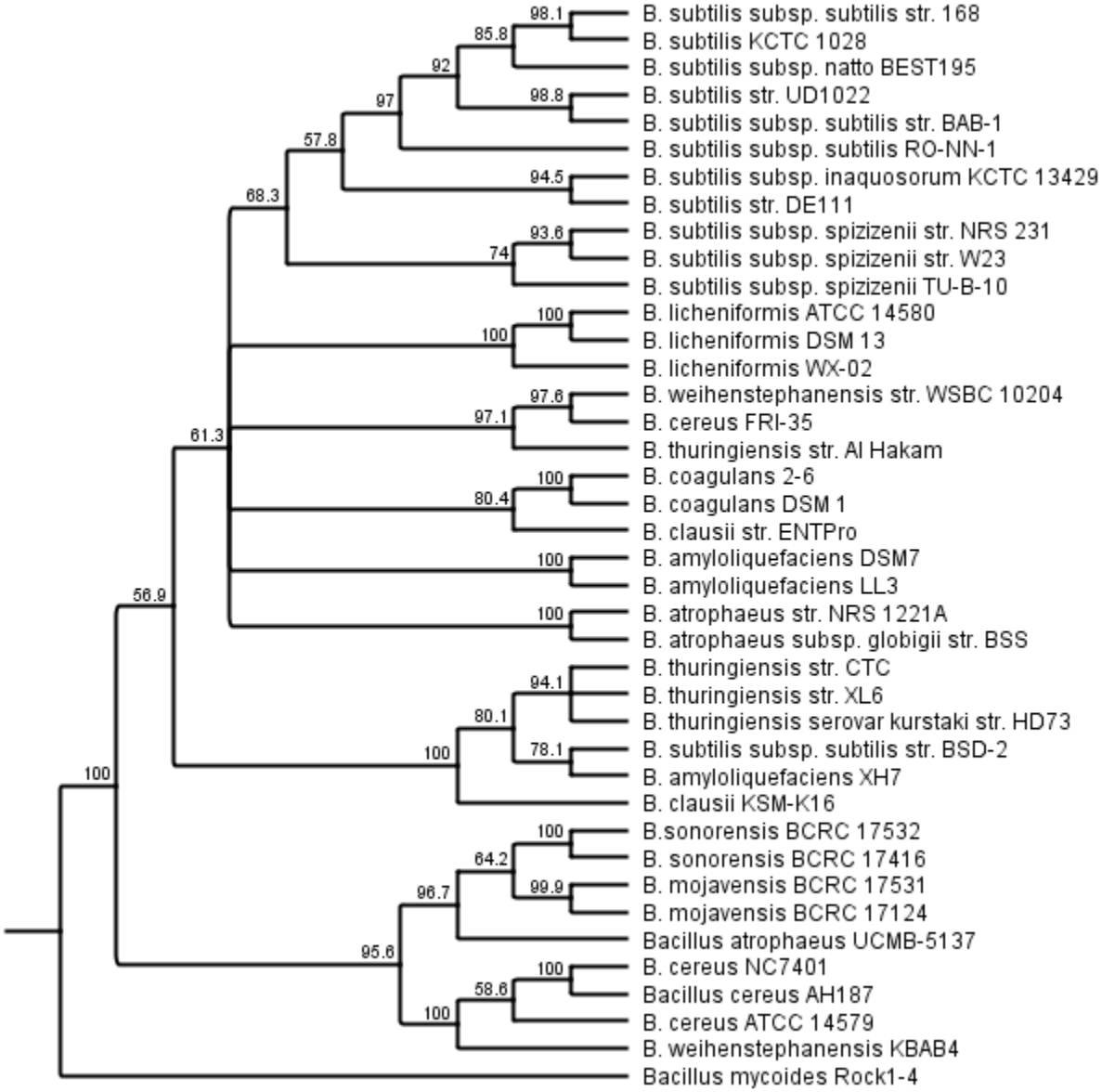
Multiple alignment using the MUSCLE algorithm (**Edgar, 2004**) was used to align the *gyrB* gene sequence of *Bacillus subtilis* DE111 with 39 other species: 38 representative species (including the six reference sequences in previous sections); and one out-group species (*Bacillus mycoides* Rock1-4). The distance matrix calculated from the alignment confirms that *Bacillus subtilis* DE111 is closest in branching and similarity to *Bacillus subtilis* subsp. *inaquosorum* str. KCTC 13429 (99%) (**Table 3**). The alignment identity percentages show that *Bacillus subtilis* DE111 is 98% similar to *B. subtilis* subsp. *inaquosorum* str. KCTC 13429 (**Figure 1**).

Figure 1. Dendrogram of multiple alignment by *gyr* gene sequence.



Phylogenetic analysis using the neighbor-joining (NJ) method (**Saitou & Nei, 1987**) placed *Bacillus subtilis* DE111 in a clade with *Bacillus subtilis* subsp *inaquosorum* str. KCTC 13429 (**Figure 2**). This confirms all previous genomic identity determinations. *Bacillus subtilis* DE111 has been placed in the *Bacillus subtilis* group and is a close relative to the *inaquosorum* subspecies.

Figure 2. Phylogenetic tree of 40 *Bacillus* species arranged in clades.



9.5 APPENDIX V- ENTEROTOXIN AND EMETIC TOXIN TESTING ON *BACILLUS SUBTILIS* DE111 BY PCR

Introduction

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

The DE111 10^9 CFU sample was 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 three were collected with a sterile loop and placed in 50mL of sterile nutrient broth in 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 broth was then transferred to a sterile 2mL centrifuge tube and spun down to pellet the bacteria. The supernatant was discarded, and the bacteria were resuspended in 180µL of lysis buffer. The enzymatic lysis buffer is a solution of Tris-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 tube of DE111 and lysis buffer is then incubated at 37°C for 30 minutes. After 30 minutes, 20µL of proteinase K and 200µL Buffer AL were added and the sample incubated at 56°C for 30 minutes. After the final incubation step, 200µL of 100% EtOH was added and the mixture inverted several times to ensure homogeneity. The DNA from this sample preparation was purified following the Qiagen protocol for purification of total DNA from animal tissues.

Primers for the toxins Hemolysin B, Non-hemolytic enterotoxin A, B, and C as well as Cytotoxin K were used (From *et al*., 2005). In addition to these, primers were acquired for the non-ribosomal lipopeptides Fengycins, Plipastatins, Surfactins, and Mycosubtilins (Tapi *et al*., 2009). A control primer for *Bacillus subtilis* was also used (Wattiau *et al*.2001). 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 each of 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; Tapi *et al*., 2009).

Results

Only one toxin amplification, *hblC* was observed during the 45 amplification cycles, as shown by the amplification plot in Figure 1. The control sample of *Bacillus subtilis* amplified in all runs and a single product was verified using a melt curve analysis following each individual test. A negative control and a postive control 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, the DE111 isolate had no significant similiarity with the *Bacillus cereus*-like toxins Table 2.

Discussion

Only one toxin displayed amplification within the 45 cycle pcr protocol. However, the genome sequence analysis found no similiarity between *hblC* and the *Bacillus subtilis* isolate. 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 nucelotide sequences in question. To confirm the results of the pcr toxin screen, genome sequence was examined for the presence of *Bacillus cereus* enterotoxins, and the results are displayed in Table 2. Additionally, the 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 anaylsis. 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. 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 DE111 was positively identified by a secondary outside lab.

Figure 1. PCR amplification plot and well setup for toxin testing

Toxins	GenBank/Accession #/FASTA	Result	Query Covered	Identical
hblC	BCU63928	No significant similarity found		
nheA	DQ885236.1	No significant similarity found		
nheB	Y19005.2	No significant similarity found		
nheC	Y19005.3	No significant similarity found		
cytK	AJ277962.1	No significant similarity found		
hblC	BCU63928	No significant similarity found		
nheA	DQ885236.1	No significant similarity found		
nheB	Y19005.2	No significant similarity found		
nheC	Y19005.3	No significant similarity found		
cytK	AJ277962.1	No significant similarity found		


Amplification Plot
Plot Type: ΔRn vs Cycle
Graph Type: Log
Plot Color: Well
Save current settings as the default
Amplification Plot
ΔRn
Cycle
Legend
A B C D E F G H
Options
Target: All
Threshold:
Auto Baseline
Show: Threshold Baseline Start: Well Target Baseline End: Well Target
View Plate Layout
View Well Table
Select Wells With:
- Select Item -
Show in Wells
View Legend
Wells: 42 Unknown 0 Standard 8 Negative Control 46 Empty
Analysis Summary:
Total Wells in Plate: 96
Wells Set Up: 50
Wells Omitted Manually: 0
Wells Flagged: 40
Wells Omitted by Analysis: 0
Samples Used: 2
Targets Used: 8

9.6 Appendix VI- ResFinder-2.1 Server Antibiotic Resistance Results

Aminoglycoside

No resistance genes found.

Beta-lactam

No resistance genes found.

Fluoroquinolone

No resistance genes found.

Fosfomycin

No resistance genes found.

Fusidic Acid

No resistance genes found.

MLS - Macrolide-Lincosamide-StreptograminB

No resistance genes found.

Nitroimidazole

No resistance genes found.

Phenicol

No resistance genes found.

Rifampicin

No resistance genes found.

Sulphonamide

No resistance genes found.

Tetracycline

No resistance genes found.

Trimethoprim

No resistance genes found.

Glycopeptide

No resistance genes found.

Selected %ID threshold: ***50.00 %***

Selected minimum length: ***60 %***

Input Files: ***DE111.fas***

Antibiotic resistance to antibiotics targeting ribosomes, gyrases, etc. 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 ResFinder antibiotic screen did not overlook any changes, a phenotypic testing for resistance in conjunction with genetic analysis was performed.

9.7 APPENDIX VII- ANTIMICROBIAL SUSCEPTIBILITY: ZONE OF INHIBITION

Antibiotic Sensitivity Testing of a Probiotic Strain *Bacillus subtilis* DE111: A Study Performed for Deerland Probiotics and Enzymes

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September 29, 2014

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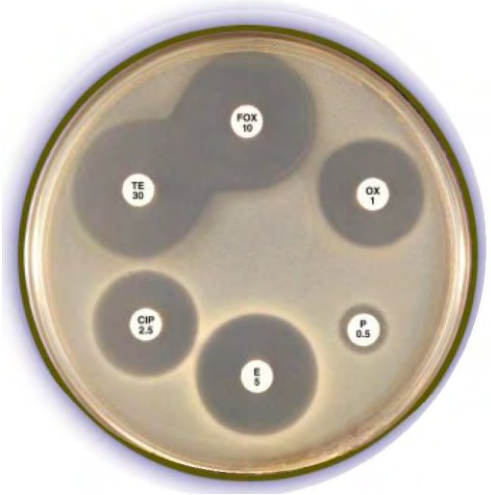
Antibiotic Sensitivity Testing

To determine if an antibiotic is effective against a particular microbe, **antibiotic susceptibility testing (AST)** is usually 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)**[2,3]. 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 *Bacillus subtilis* strain 08683 to twenty-two different antibiotics.

Kirby-Bauer Method

The Bauer, Kirby, Sherris and Turck method[1], commonly called the **Kirby-Bauer, Bauer-Kirby** or **filter disk diffusion method**, allows microbiologists to test the 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 Müeller-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[3]. For each antibiotic, the test organism is reported as being **susceptible (S)**, **intermediate (I)**, or **resistant (R)** to the antibiotic.

Figure 1. Zones of inhibition around antibiotic disks by the Kirby-Bauer Method.



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."[3]

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

Bacillus subtilis strain 08683 was submitted by Deerland Enzymes, Inc (3800 Cobb International Blvd NW, Kennesaw, GA 30152; www.deerland-enzymes.com) 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[2] 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 and bacitracin). 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)
Cyclic polypeptide:
bacitracin (B-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[2,3]. *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 35ºC. 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 35ºC) 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 35ºC 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 x 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 $BaCl_2 \cdot 2H_2O$) to 99.5 mL of 0.18 M H_2SO_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 surfaces 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. The plates were incubated 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. 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.

If a zone of inhibition was present and measured, interpretation of the measurement required the use of the table on the manufacturer's package insert for the BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs or tables 2A-2J in the CLSI M-100-S24[3] for the Oxoid Antimicrobial Susceptibility Test Discs.

Results

Table 2. Measured diameters for the zones of inhibition around 23 different antibiotics for *Bacillus subtilis* 08683. 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.

	Zone of Inhibition (mm) / Interpretation(s)				
Antibiotic	*B. subtilis* 08683[a]		*S.aureus* 25923 [b]	*E. coli* 25922[b]	*E. coli* 35218[b]
Aminoglycosides					
Gentamicin, GM-120	36	S	Y	Y	.
Kanamycin, K-30	30	S	Y	Y	
Neomycin, N-30	20	S	Y	Y	
Streptomycin, S-300	19	S	Y	Y	
Beta-lactams- penicillins:					
Ampicillin, AM-10	26	S, R	N	Y	Y
Amoxicillin/clavulanic acid, AmC-30	21	S	Y	Y	Y
Penicillin, P-10	28	S, R	Y		Y
Beta-lactams- cephalosporins:					
Cefaclor, CEC-30	28	S	Y	Y	
Cephalothin, CF-30	27	S	Y	Y	
Ceftriaxone, CRO-30	24	S, R	Y	Y	
Cefotaxime, CTX-30	25	S, R	Y	Y	
Beta-lactams- carbapenems:					
Imipenem, IPM-10	48	S		Y	
Fluoroquinolone:					
Ciprofloxacin, CIP-5	30	S	Y	Y	
Fosfomycin:					
Fosfomycin, FOS-200	26	S	Y	Y	
Macrolide-Lincosamide-Streptogramin B					
Erythromycin, E-15	25	S	Y		
Clindamycin, CC-2	26	S	Y		

Quinupristin/dalfopristin, QD-15	20	S	Y		
Phenicol					
Chloramphenicol, C-30	24	S	Y	Y	
Rifampicin					
Rifampin, RA-5	22	S	Y	Y	
Sulphonamide					
Sulfamethoxazole-trimethoprim, SXT-23.75/1.25	32	S	Y	Y	
Tetracycline:					
Tetracycline (Te-30)	30	S	Y	Y	
Trimethoprim					
Trimethoprim (TMP-5)	24	S	Y	Y	
Glycopeptide:					
Vancomycin (Va-30)	21	S	Y		

[a]S = susceptible, I = intermediate, R = Resistant.
[b]Diameter 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 *Bacillus subtilis* 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 *Bacillus subtilis* would be allied. Because *Bacillus 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 *Bacillus subtilis* 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 *Bacillus subtilis* was clearly susceptible to 1st (cefaclor) and 2nd (cephalothin) generation cephalosporins, results were mixed for 3rd 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 *Staphylococcus aureus* control. Phylogenetic analyses place *Bacillus subtilis* closer to the staphylococci than to the streptococci or enterococci (Ahmad *et at*., 2000) ; however, these analyses do not account for differences in antibiotic resistance or sensitivity mechanisms. The enhanced resistance of *Bacillus subtilis* to β-lactams (in comparison to *Staphylococcus 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 *Bacillus 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 *Bacillus subtilis.*

Based on the zones around the remaining antibiotics, *Bacillus subtilis* 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/dalfoprisitn (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.

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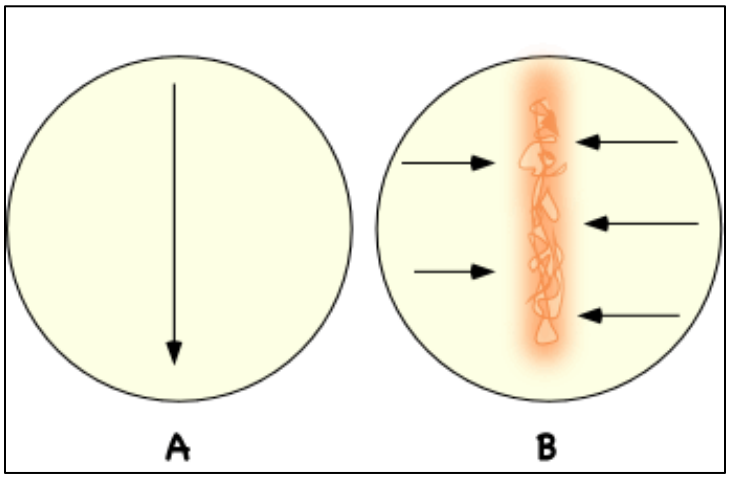
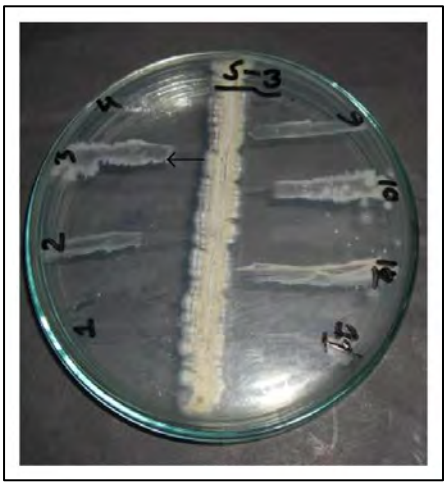
Screening for Production of Inhibitory Compounds from *Bacillus subtilis*.

Bacillus subtilis is able to produce a variety of antibiotics including peptides that are either ribosomally synthesized or non-ribosomally generated, as well as some non-peptide compounds[1]. To determine if *Bacillus subtilis* 08683 produces any antimicrobial compounds, a cross-streak experiment was performed. For this approach, *Bacillus subtilis* was inoculated onto an agar surface using a straight central line as shown in figure 2A. The plate was incubated for 12-16 hours at 35°C to allow for growth and secretion of antimicrobial compounds. Test organisms, listed below, were then inoculated onto the plate perpendicular to the central streak line as shown in figure 2B. The test organisms are inoculated up to the area of central growth, but not through or into the central growth. The plate was incubated at 35°C for 24 hours.

Figure 2. A simple screening assay for production of antimicrobial compounds. The diagram to the left illustrates the cross-streak inoculation method for the screening of antimicrobial compound production. The photograph to the right is an example of the growth patterns demonstrating inhibition and no inhibition of growth by a compound produced by the central test organism.





After incubation, the growth of the test organisms was observed. Test organisms that grow up to the central organism indicate that the central organism does not produce a compound that inhibits their growth. However, growth of a test organism that stops at a distance from the central organism is indicative that an inhibitory compound is produced by the central organism.

This approach was used to test the following organisms against *Bacillus subtilis*: ***Escherichia coli*** ATCC 35218 (β-lactamase positive) and ATCC 25922 (β-lactamase negative); ***Pseudomonas aeruginosa*** ATCC 39324 and ATCC 29260; ***Enterococcus faecalis*** ATCC 19433 and ATCC 33186; ***Staphylococcus aureus*** ATCC 25923 (methicillin-sensitive), ATCC 29213 (methicillin-sensitive), ATCC1688 (methicillin-resistant), ATCC BAA 41 methicillin-resistant); ***Staphylococcus epidermidis*** ATCC 49134; ***Staphylococcus saprophyticus*** ATCC 15305; ***Staphylococcus pseudintermedius*** (dog isolate, methicillin resistant); and ***Candida albicans*** ATCC 18804.

Of the organisms tested, **ALL** staphylococci species were inhibited. No inhibition was observed for the other test bacteria (the enterococci, pseudomonads and *Escherichia coli*) or the yeast *Candida albicans*.

The specificity for the staphylococci warrants further investigation, especially in light of the results showing that methicillin resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* were inhibited. These results did not vary when using trypticase soy agar or Müeller-Hinton agar.

As mentioned, *Bacillus subtilis* is able to produce a variety of antibiotics. Which of these compounds is exerting the inhibitory effect on staphylococci is unknown, but maybe worth the effort to investigate. *Bacillus subtilis* is a known producer of bacitracin. Bacitracin is effective against several Gram-positive bacteria, but its application is topical rather than internal due to its toxicity. PCR detection of bacitracin-related genes can be accomplished by ampification of bacitracin synthetase genes (*bacA*, *bacB*, and *bacC)* and the bacitracin transporter gene cluster (i.e., *bcrA*, *bcrB*, and *bcrC*)[2]. Because the strains of *Enterococcus faecalis* used in this study are bacitracin sensitive by Kirby-Bauer AST but were resistant to the *Bacillus subtilis* inhibitory factor by the cross-streak method, it is unlikely that bacitracin is the inhibitory substance produced by *Bacillus subtilis*. Lack of bacitracin synthesis in this strain of *Bacillus subtilis* requires further confirmation. In a somewhat recent study that investigated *Bacillus subtilis* inhibition of *Staphylococcus aureus*, it was reported that production of the natural cyclic lipopeptide antibiotics surfactin and plipastatin from *Bacillus subtilis* inhibited, at least in part, growth and virulence factor expression of *Staphylococcus aureus*[3]. It was not mentioned whether or not the inhibitory effects occurred in other species of staphylococci or were limited to staphylococci.

Literature Cited

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9.8 Appendix VIII-Antimicrobial Susceptibility: Minimal Inhibitory Concentration

Protocol for Micro-Dilution Assay

- Initial Dilutions
  - 1 Sensi-Disc was combined with 1mL of Butterfields Phosphate Buffer (PBS)
  - Rifampin: 0.0128mg was combined with 10mL of PBS
  - Penicillin/Streptomycin/Neomycin: 100µL was combined with 900µL of PBS
- Serial Dilutions
  - Various (1:1) dilutions were made down the rows on the microplate
- Plate Contents
  - 100µL of antibiotic solution
  - 50µL of tryptic soy broth
  - 50µL of DE111 liquid culture
- Optical Density Readings (615nm)
  - 0 Hours
  - 16 Hours
- Delta from the two time points were taken to determine MIC
  - Susceptible: ≤ 4µg/mL
  - Intermediate: 8-16µg/mL
  - Resistant: ≥ 32µg/mL

Cockerill, F.R., Wilker, M.A., Alder, Jd., Dudley, M.N., Elipoulos, G.M., Ferraro, M.J., Hardy, D.J., Hecht, D.J., Hindler, J.A., Patel, J.A., Powell, M., Swenson, J.M., Thompson, R.B., Traczewski, M.M., Turnidge, J.D., Weinstein, M.P., and Zimmer, B.L. (2012). Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard-Eleventh Edition. *Clinical and Laboratory Standards Institute M02-A11*. 32(1); 1-58.

Table 1. Antibiotics Tested

Antibiotic Name	Antibiotic Code	Concentration (µg/mL)
Gentamicin	GM-120	120
Kanamycin	K-30	30
Neomycin	N-30	30
Streptomycin	S-30	30
Ampicillin	AM-10	10
Amoxicillin/Clavulanic Acid	AmC-30	30
Cefaclor	CEC-30	30
Cephalothin	CF-30	30

Note: all antibiotics were Sensi-Discs unless otherwise specified.
**indicates a powdered form*

Ceftriaxone	CRO-30	30
Cefotaxime	CTX-30	30
Imipenem	IPM-10	10
Ciprofloxacin	CIP-5	5
Fosfomycin	FOS-200	200
Erythromycin	E-15	15
Clindamycin	CC-2	2
Quinupristin/Dalfopristin	QD-15	15
Chloramphenicol	C-30	30
Rifampin*	RA-5	5
Sulfamethaxole-Trimethoprim	SXT-23.75/1.25	23.75/1.25
Tetracycline	Te-30	30
Trimethoprim	TMP-5	5
Vancomycin	Va-30	30
Penicillin	P-10	6 (10IU)

Table 2. Micro-dilution assay MIC results

Antibiotic	Concentration (µg/mL)	MIC (µg/mL)	S, I, or R
Gentamicin	120	< 0.5	S
Kanamycin	30	< 0.2	S
Neomycin	30	< 1	S
Streptomycin	30	< 2.5	S
Ampicillin	10	< 0.5	S
Amoxicillin/Clavulanic Acid	30	< 0.2	S
Cefaclor	30	< 0.2	S
Cephalothin	30	< 0.1	S
Ceftriaxone	30	0.2	S
Cefotaxime	30	< 0.2	S
Imipenem	10	< 0.1	S
Ciprofloxacin	5	< 0.1	S
Fosfomycin	200	< 1	S
Erythromycin	15	< 0.06	S
Clindamycin	2	< 1	S
Quinupristin/Dalfopristin	15	0.2	S
Chloramphenicol	30	< 0.2	S

Rifampin*	5	< 0.1	S
Sulfamethaxole-Trimethoprim	23.75/1.25	0.5	S
Tetracycline	30	< 0.1	S
Trimethoprim	5	0.2	S
Vancomycin	30	< 0.2	S
Penicillin	6	< 0.1	S

Minimum Inhibitory Concentrations (MICs) were measured and interpreted as resistant, intermediate, or susceptible to the antibiotic in question. Measurement of pure *Bacillus subtilis* DE111 cultures were grown as a positive control to reference MIC baselines. Blanks imply testing is not done or no control range has been published. *Bacillus subtilis* DE111 was susceptible to all of the antibiotics screened in the micro-dilution assay.

- Susceptible (S): ≤ 4μg/mL
- Intermediate (I): 8-16μg/mL
- Resistant (R): ≥ 32μg/mL

9.9 APPENDIX IX- *BACILLUS SUBTILIS* EFSA QPS STATUS

Extracted from: The EFSA Journal (2007) 587, 1-16; Additional Reference: The EFSA Journal (2008) 923, 16-48

2.2.1. Bacillus

Safety concerns linked to the presence of *Bacillus* spp in the food chain come mainly from the ability of some strains belonging to several species of the genus to cause foodborne diseases characterized by emesis and/or diarrhoea. Foodborne disease is mostly caused by strains of *Bacillus cereus* which was therefore not included in the QPS list (EFSA 2007a). More rarely, other species of *Bacillus* cause foodborne disease. Several virulence factors have so far been identified and include a range of proteins or peptides with enterotoxic or cytotoxic activities, produced by the bacterial cells in the foods or in the intestine of the host. Absence of the ability to produce these toxins was proposed as the basis of the qualification for QPS for *Bacillus* spp. other than *B. cereus* in the previous EFSA opinion (EFSA 2007a, Appendix B).

Safety concerns for *Bacillus* spp. also arise from various, uncommon but severe infections, different in their symptoms from foodborne poisoning, either systemic infection or restricted

to various organs. In its previous opinion on QPS (EFSA 2007a, Appendix B), EFSA did not consider that these infections were linked to the presence of the bacteria in the food chain. The previous opinion also recognised the high prevalence of *Bacillus* spp in various environments, independent of their intentional introduction in the food chain.

Toxins from Bacillus species not belonging to the B. cereus group

In the previous EFSA QPS opinion, some *Bacillus* species not belonging to the *B. cereus* group were granted QPS status but absence of toxigenic activity was a qualification: "Absence of emetic toxin with surfactant activities" and "absence of enterotoxic activities". Results published since the preparation of the opinion give more indications on the nature of some of the toxic compounds produced by these QPS *Bacillus* species: Amylosin produced by *B. amyloliquefaciens*, a member of the *B. subtilis* group (Mikkola et al., 2007); the lipopeptides fengycin and surfactin from *B. subtilis* and *B. mojavensis* (Huang et al., 2006, From et al., 2007a); pumilacidin from *B. pumilus* (From et al., 2007b); lichenysin from *B. licheniformis* (Nieminen et al., 2007). Pumilacidin was associated with a food borne poisoning outbreak linked to rice (From et al., 2007b). Lichenysin was produced by *Bacillus* sp. isolated from mastitis. Surfactin was proposed to be the origin of the cytotoxic activities found in some strains of *B. mojavensis* implicated in foodborne poisoning (From et al., 2007a). However, this strain also produced amylopsin, which had the major contribution to toxicity according to Apetroaie-Constantin et al. (2008).

All the above described toxins are peptides with toxic activities on cell lines and sperm cells similar to that of the emetic toxin of *B. cereus.* They can be detected by the same biological tests, but their ability to cause emesis has not been proven. Therefore the qualification in the list of QPS granted micro-organisms "absence of emetic food poisoning toxin with surfactant activities" for *Bacillus* strains should be reworded as "absence of food poisoning toxins, absence of surfactant activities". The approach proposed in the previous QPS opinion (EFSA 2007a, Appendix B) would permit to detect these toxic peptides and the strains producing them would not be qualified for the QPS.

All these toxic peptides had toxic activities on cells similar to the emetic toxin of *B. cereus*. Although this emetic toxin usually causes mild poisoning, it has been responsible for severe and fatal liver failures (Mahler et al. 1997; Posfay-Barbe 2008; Dierick et al. 2005).

Conclusion concerning the *Bacillus* species:

- No change is made to the QPS list for *Bacillus* species as previously defined (EFSA, 2007a) (see Table 4).

- The qualification concerning QPS *Bacillus* species is modified to "Absence of food poisoning toxins, absence of surfactant activities, absence of enterotoxic activities".

9.10 APPENDIX X- MEM ELUTION TEST FOR CYTOTOXICITY OF *BACILLUS SUBTILIS* DE111

NELSON LABORATORIES

Sponsor:
Maggie Leroux
Deerland Enzymes
3800 Cobb Intl. Blvd.
Kennesaw GA 30152

MEM Elution Final Report

Test Article: LB13.768 lot 06234
Laboratory Number: 685418A.1 Amended
Study Received Date: 08 Apr 2013
Study Completion Date: 22 Apr 2013
Test Procedure(s): Standard Test Protocol (STP) Number: STP0032 Rev 08

Summary: The Minimal Essential Media (MEM) Elution test was designed to determine the cytotoxicity of extractable substances. An extract of the test article was added to cell monolayers and incubated. The cell monolayers were examined and scored based on the degree of cellular destruction. All test method acceptance criteria were met.

Results:

Test Article:

Results Pass/Fail	Scores				Extraction Ratio	Amount Tested / Extraction Solvent Amount
	#1	#2	#3	Average		
Pass	0	0	0	0	0.1 g/mL	2.1 g / 21 mL

Controls:

Identification	Scores				Extraction Ratio	Amount Tested / Extraction Solvent Amount
	#1	#2	#3	Average		
Negative Control - Polypropylene Pellets	0	0	0	0	0.2 g/mL	4 g / 20 mL
Media Control	0	0	0	0	N/A	20 mL
Positive Control - Latex Natural Rubber	4	4	4	4	0.2 g/mL	4 g / 20 mL

Acceptance Criteria: The United States Pharmacopeia & National Formulary (USP 87) states that the test article meets the requirements, or receives a passing score **(Pass)** if the reactivity grade is not greater than grade 2 or a mild reactivity. The ANSI/AAMI/ISO 10993-5 standard states that the achievement of a numerical grade greater than 2 is considered a cytotoxic effect, or a failing score **(Fail)**.

Nelson Laboratories acceptance criteria was based upon the negative and media controls receiving "0" reactivity grades and positive controls receiving a 3-4 reactivity grades (moderate to severe). The test was considered valid as the control results were within acceptable parameters.

(b) (6)

Study Director

Bobbi L. Rushton-Castro

ACLASS TESTING

07 July 2013

Amended Report Date

mlb

The cell monolayers were examined microscopically. The wells were scored as to the degree of discernable morphological cytotoxicity on a relative scale of 0 to 4:

Conditions of All Cultures	Reactivity	Grade
No cell lysis, intracytoplasmic granules.	None	0
Not more than 20% rounding, occasional lysed cells.	Slight	1
Not more than 50% rounding, no extensive cell lysis.	Mild	2
Not more than 70% rounding and lysed cells.	Moderate	3
Nearly complete cell destruction.	Severe	4

The results from the three wells were averaged to give a final cytotoxicity score.

Procedure: The amount of test material extracted was based on ANSI/AAMI/ISO and USP surface area or weight recommendations. Test articles and controls were extracted in 1X Minimal Essential Media with 5% bovine serum for 24-25 hours at 37 ± 1°C with agitation. Multiple well cell culture plates were seeded with a verified quantity of industry standard L-929 cells (ATCC CCL-1) and incubated until approximately 80% confluent. The test extracts were filtered per sponsor request and added to the cell monolayers in triplicate. The cells were incubated at 37 ± 1°C with 5 ± 1% CO_2 for 48 ± 3 hours.

Pre and Post Extract Appearance		
Test Articles	Pre extract	Clear with no particulates present
	Post extract	Clear with no particulates present pH shift-acidic
Controls	Pre extract	Clear with no particulates present
	Post extract	Clear with no particulates present No color change noted

Amendment Justification: At the request of the sponsor, the initial report was separated into individual reports by test article. A sentence was removed from the first paragraph of the procedure section to more accurately reflect the testing performed.

9.11 APPENDIX XI- AFRICAN GREEN MONKEY (ATCC CCL-81) *IN VITRO* CYTOTOXICITY OF *BACILLUS SUBTILIS* DE111

Emery Pharma Services, *Emeryville CA, USA*

Study Report

Sponsor:

Deerland Enzymes, Inc.

Investigators:

Kiran Bijlani, Ph.D. and Hubert Lin, M.S.

Emery Pharma Services

5980 Horton St, Suite 575

Emeryville, CA 94608

Report Title:

Cytotoxicity of Probiotic Test Article using Vero cell line

Emery Pharma Services Study #:

03-04-2016-EPS

Date for initiation of study:

March 22, 2016

Date for completion of study report:

April 21, 2011

Signature page

Authors: Kiran Bijlani and Hubert Lin

I have read this report and confirm that to be the best of my knowledge it accurately describes the conduct and results of the study.

(b) (6)

Kiran Bijlani
Investigator

05/10/2016
Date

(b) (6)

Hubert Lin
Investigator

5/10/16
Date

Emery Pharma Services
5980 Horton Street, Suite 575
Emeryville, CA 94608

Introduction

Deerland Probiotics and Enzymes contracted Emery Pharma Services (EPS) to determine *in vitro* cytotoxicity (CT50) of Test Articles *Bacillus subtilis* DE111 using Vero cell line.

Materials and Methods

Test Articles

Deerland Probiotics and Enzymes provided one Test Article (*Bacillus subtilis* DE111, LB15.162). Test Article was sent as a dry powder and it was reconstituted overnight by suspending 1mg powder in 99 mL of Tryptic Soy Broth (TSB). The test article culture was used as is or passed through a filter and the filtrate was used for further experiments. The positive control used in the study was 0.01% pure hypochlorous acid.

Cell Lines

Vero cells (CCL-81) were obtained from the American Type Culture Collection (Manassas, VA). The strain was stored in liquid nitrogen until ready for use. The cell line was prepared by thawing a 1 mL vial of the cell line and splitting it into 2 T-75 flasks with 15 mL of RPMI 1640 containing L-glutamine, 4.5 g/L of glucose, 1 mM sodium pyruvate, 10% fetal bovine serum and 100 IU/mL of Penicillin-Streptomycin solution. Cells were maintained and incubated in a humidified chamber set at 37°C with 5% CO2. Cells were split one day prior to usage.

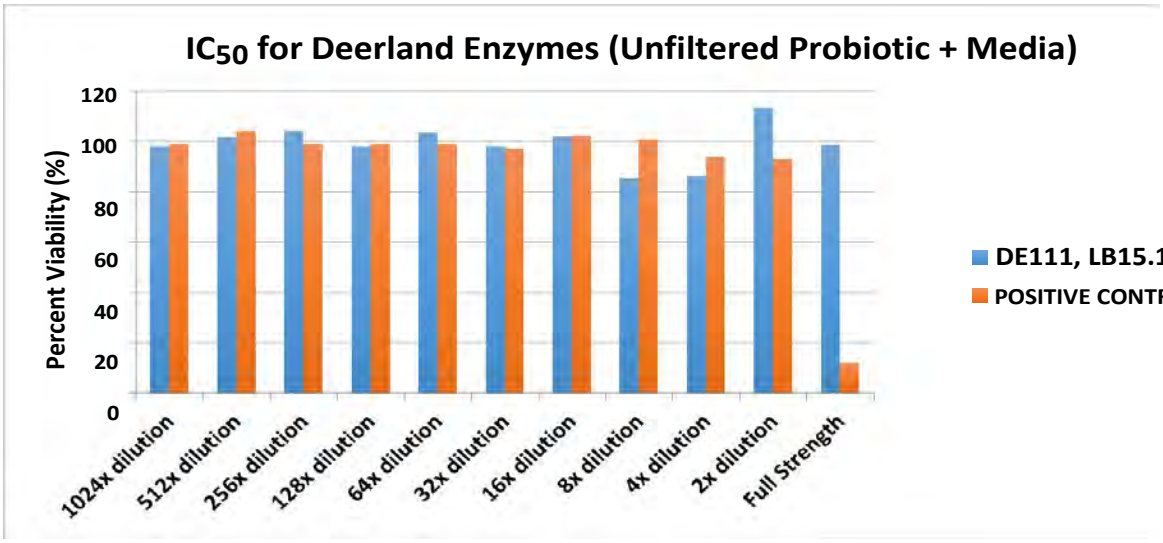
Cytotoxicity Assay

The Vero cells were counted using trypan blue and a hemocytometer. 100 µL of 2.0 x 10^6 cells/mL were added to each well of the 96-well microtiter plate and incubate for 24 hours in media. Test Article was reconstituted and cultured overnight at 37°C by dissolving 1mg of Test Article in 99 mL TSB. The culture was then grown until an OD650 reached 0.4 before use. Two-fold serial dilutions were performed in 11 wells (the last well was a blank control) with the full culture along with control drug. The media was aspirated from each well of cell plate and 170 µL of test articles + 30 µL media were added to each well. After 24 hours incubation in test articles the wells were aspirated and 200 µL media was added to each well. Cell viability was determined using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) kit. The optical density (OD) was read using BioTek Synergy 2 at 490 nm. Cytotoxicity (CT50) was calculated by plotting percent viability (determined in respect to blank control) versus log of the compound dilutions. All experiments were performed in triplicate.

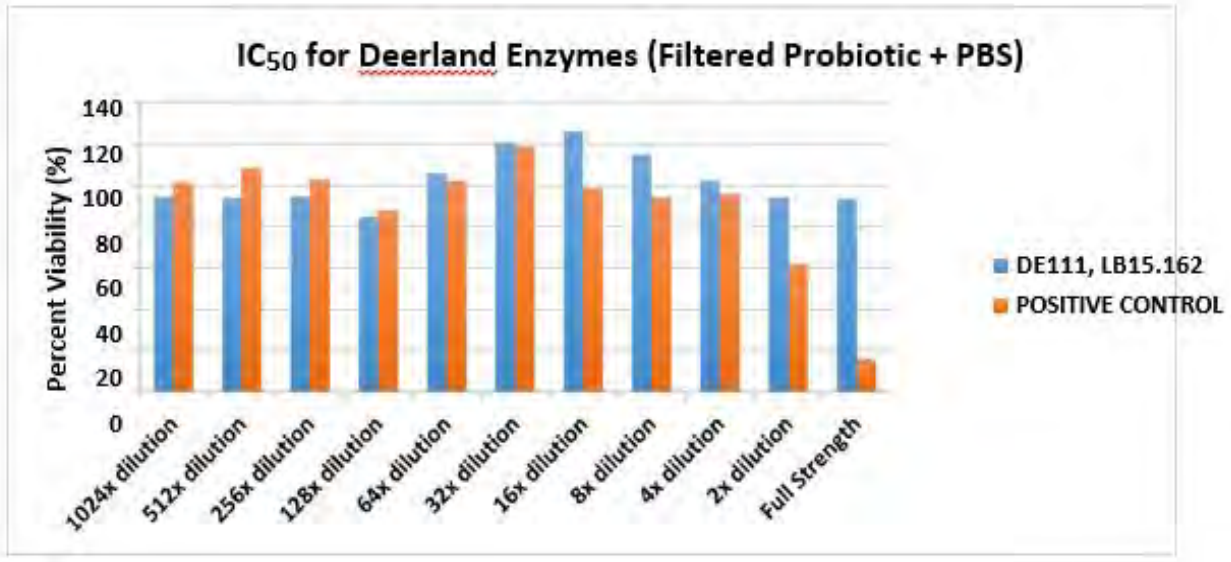
Results

Two assay conditions were tested: unfiltered Test Article culture diluted in media (shown in Graph 1), and filtered Test Article culture diluted in PBS (shown in Graph 2). Both conditions were compared using appropriate controls. The cytotoxicity data is as follows (reported in percent viability):

Graph 1: CT_{50} for unfiltered Test Article incubated Media



Graph 2: CT_{50} for filtered Test Article incubated in PBS



Discussion and Conclusion

Two assay conditions were tested, in presence or absence of bacteria in the probiotic test article. This is to account for the presence of bacteria in case it had a false positive effect on the CellTiter assay. In both conditions Test Article did not show cytotoxic effects in Vero cell lines, therefore we are unable to determine the CT50 of the Test Article.

References

Ammerman NC, Beier-Sexton M, and Azad AF. 2008. Growth and maintenance of Vero Cell. *Current Protocols in Microbiology*, 1-10.

9.12 Appendix XII- Zones of Hemolysis and Boar Sperm Motility Assay

University of Wisconsin-Madison, Madison WI, USA

Ex. 2 of Deerland Probiotics and Enzymes Project
Isolate cereulide toxin in 0.2 mL of methanol for testing in boar sperm motility assay

April 12, 2016

- One cryocare bead of each strain into a 15mL conical tube filled with 10mL of Difco nutrient broth (BD #234000; lot #5296749)
- Tubes were lightly sealed (opened enough for air to diffuse) and placed in an incubator (30*C) with shaking at ~20rpm
- Cultures were allowed to grow overnight
    - started at 9:15am

April 13, 2016

- Overnight growth of cultures (~24 hours) were transferred to a fresh 15mL conical tube (to get rid of the Cryocare bead)
- Cultures were pelleted at 1800g for 10min
- Cultures were washed with D-PBS and pelleted again at 1800g for 10min
- Pellets were re-suspended in 1mL of D-PBS and absorbance read at 600nm

Table 1. Absorbance readings of *Bacillus cereus* 14579 and *Bacillus subtilis* DE111 at 600 nm

Strain	OD 600
DE111	0.4776
14579	1.1261

- Each culture was serially diluted and plated to blood agar plates as follows
    - plates were placed into 30*C incubator overnight

April 14, 2016

- Blood agar plates were removed from the incubator and placed at room temperature in the biosafety cabinet
- Images were taken of each plate

Figure 1 *Bacillus cereus 14579*

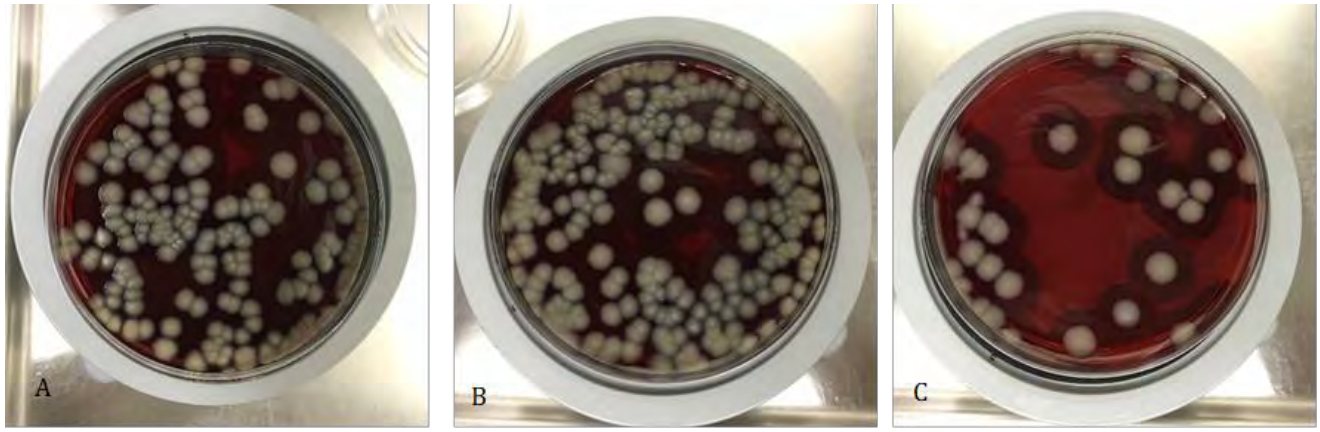
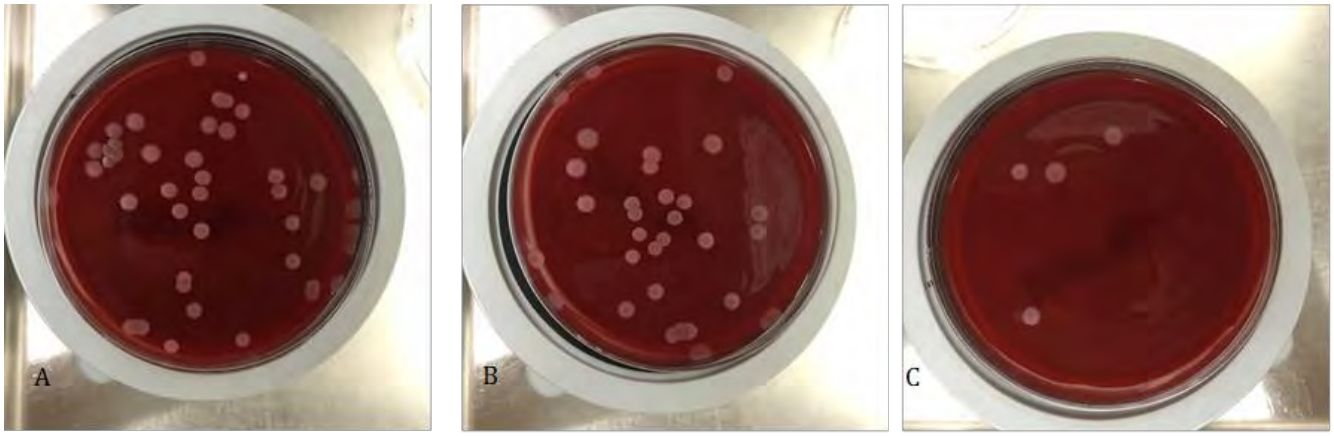


Figure 2 *Bacillus subtilis* DE111



Note: according to Anderson et. al. 2004, a wide zone of hemolysis (3 - >/= 4 mm) indicates a non-cereulide producing strain; whereas a small zone of hemolysis (</= 2 mm) may be indicative of a cereulide producing strain. In this experiment, 14579 is indicative of a cereulide producing strain, whereas DE111 is a non-cereulide producing strain.

Table 2. Counts of *Bacillus cereus* 14579 and *Bacillus subtilis* DE111 on blood agar plates

Plate	14579	DE111
A	189	33
B	257	44
C	37	4

- When considering dilution factors
  - 2.72e8 CFU/mL for 14579
  - 3.9e7 CFU/mL for DE111
- Using a sterile inoculating loop, a loopful of bacteria colonies (~5-10ug) from each strain was placed into 0.2mL of 100% methanol in a polypropylene vial
- The polypropylene vial was sealed with a screw cab fitted with a rubber gasket
- The vials were placed into a well of a heating block that was filled with water and brought to 100°C for 15min
- After the vial was heated, the vials were cooled and then stored at 4°C for later use

April 15, 2016 – Boar sperm motility

Table 3. Baseline at 30 minutes after semen warmup to 37°C

Condition	Total Motility %	Progressive Motility %	Total Sperm Evaluated
Baseline	86	49	681

Following evaluations were done at 15 minutes of further incubation at 37°C. 3 µl of sample or methanol was added as higher amounts of methanol interfered with sperm motility. Heated and non-heated methanol was also tested in case there was an effect of heating.

Table 4. Total motility, progressive motility and total sperm evaluated using various test conditions

Condition	Total Motility %	Progressive Motility %	Total Sperm Evaluated
No addition	90	62	811
Methanol	86	57	697
Heated Methanol	92	59	879
Valinomycin 30 ng	28	12	322
Valinomycin 3 ng	33	14	574
Bacillus subtilis DE111	89	56	635
Bacillus subtilis DE111	83	51	786
No addition repeat	83	50	1117

The impact of valinomycin and lack of an effect of *Bacillus subtilis* DE111 on the total motility of boar sperm is observed in table 4.

9.13 APPENDIX XIII- ALLERGEN POTENTIAL OF *BACILLUS SUBTILIS* DE111

Bacillus subtilis has been used in food processes for many years and has generated no known safety concerns.

Despite this lack of general concern, the potential that *Bacillus subtilis* DE111 could be a food allergen was assessed by comparing the whole genome sequence (GenBank: CP013984.1) against sequences of known allergens. Based on the sequence homology alone, it was concluded that the *Bacillus subtilis* DE111 is unlikely to pose a risk of food allergenicity.

The most current allergenicity assessment guidelines developed by the Codex Commission (2009) and Ladics et al. (2011) recommend the use of FASTA or BLASTP search for matches of 35% identity or more over 80 amino acids of a subject protein and a known allergen. Ladics et al. (2011) further discussed the use of the "E-score or E-value in BLAST algorithm that reflects the measure of relatedness among protein sequences and can help separate the potential random occurrence of aligned sequences from those alignments that may share structurally relevant similarities." High E-scores are indicative that any alignments do not represent biologically relevant similarity, whereas low E-scores ((10-7) may suggest a biologically relevant similarity (i.e., in the context of allergy, potential cross reactivity). They suggest that the E-score may be used in addition to percent identity (such as > 35% over 80 amino acids) to improve the selection of biologically relevant matches. The past practice of conducting an analysis to identify short, six to eight, contiguous identical amino acid matches is associated with false positive results and is no longer considered a scientifically defensible practice.

The Codex Commission states:

> "A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens."

The search for 80-amino acid stretches within the *Bacillus subtilis* DE111 whole genome sequence with greater than 35% identity to known allergens using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database (http://www.allergenonline.org/index.shtml). The database containing 1897 (version released Jan 12, 2015) peer-reviewed allergen sequences (listed in http://www.allergenonline.org/databasebrowse.shtml) revealed multiple stretches throughout the genome sequence with over 35% identity (links are provided as results file is too large to include).

Although cautioned against in Codex (2009), researched by Herman *et al.* (2009) and further elaborated by Ladics *et al.* (2011) and on AllergenOnline.org that there is no evidence that a short contiguous amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (35%), this database does allow for isolated identity matches of 8 contiguous amino acids to satisfy demands by some regulatory authorities for this precautionary search. Performing this search produced no sequence matches with known allergens.

In conclusion, based on the sequence homology alone, *Bacillus subtilis* is unlikely to pose a risk of food allergenicity.

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9.14 APPENDIX XIV - THE EFFECT OF *BACILLUS SUBTILIS* DE111 ON THE DAILY BOWEL MOVEMENT PROFILE FOR PEOPLE WITH OCCASIONAL GASTROINTESTINAL IRREGULARITY

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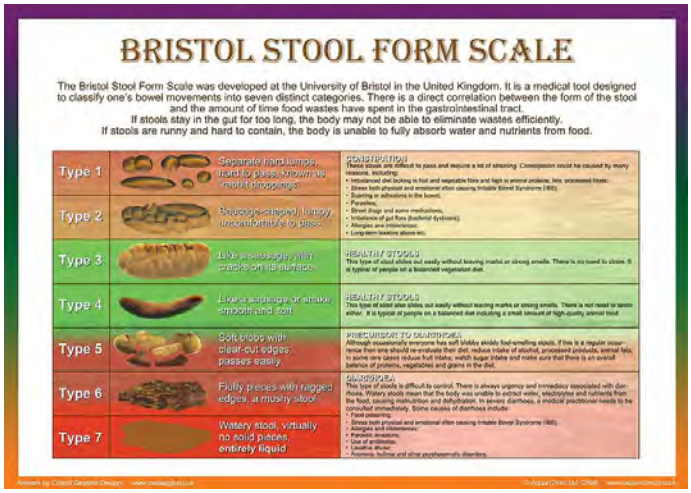
Abstract- *Bacillus subtilis probiotics have been shown to influence several aspects of the human gut including motility, epithelial strength, inflammation, etc. that may change bowel movement frequency and/or type. To explore the efficacy of Bacillus subtilis (Bacillus subtilis) DE111 in this regard, 50 people were evaluated by their stool profile, food diary and questionnaire while taking a probiotic or placebo daily over the course of 105-days. The stools were scored based on the Bristol Stool Chart index. Statically significant results showed those in the DE111 group moved to a healthier bowel index while those in the Placebo group stayed the same, providing evidence that DE111 may improve occasional constipation and/or diarrhea.*

I. INTRODUCTION

People who have at least one bowel movement per day and pass good textured feces (not too hard or soft) are considered to have 'normal' bowel function. However, occasional constipation and/or diarrhea can be a burdensome gastrointestinal issue that occurs in many individuals and whose treatment remains challenging. Some health professionals use the Bristol Stool Chart to classify stool type as it may be difficult to differentiate between normal and abnormal stools. This scale can help assess the consistency and the time stools spent in the bowels. Type 1 stools have spent the longest time, while type 7 stools spend the least time (Refer to Figure 1 for the Bristol Stool chart used in this study).

Figure 1. Bristol Stool chart.



People whose stools are classified as type 1 or type 2 are individuals who suffer from constipation. This gastrointestinal discomfort can be the result of many factors including a poor diet, excess stress or the normal aging process. Constipation can often be caused by acute dysbiosis which is common for those that are on antibiotic treatment or low fiber diets. When there is a balance in the normal flora of the gut, beneficial bacteria can hold water facilitating the passing of feces. When absent, the stools lack a normal amorphous quality and become formed lumps which can be hard and abrasive. The typical diameter of these lumps can range from 1 to 2 cm, and can be painful to pass due to their hard and scratchy nature. On the other hand, people who fall within the 5-7 stool range may have a hyperactive colon (fast motility) or excess dietary potassium. These people may suffer from sudden dehydration or stress related spikes in blood pressure; both conditions can cause the rapid release of water and potassium from blood plasma into the intestinal cavity. In addition, stools in this range are indicative of a hypersensitive personality

prone to stress, diets rich in spices, fats, high mineral contents and the use of osmotic (mineral salts) laxatives. Probiotic supplementation of the intestinal microflora may promote healthy intestinal homeostasis.

The *Bacillus* species are rod-shaped, spore-forming, aerobic, gram-positive bacteria that are ubiquitous in nature. There is evidence that *Bacillus subtilis* might be a part of the normal gut flora of humans. Some human intestinal biopsy samples have shown that subtilis does populate the gut in humans as normal human intestinal flora (6). *Bacillus subtilis* has been used abundantly in traditional ethnic food processing in East Asia. Natto, in particular, is a cheese-like food, processed by inoculating soaked and steamed soybeans with live *Bacillus* from rice straw [1, 2]. Although the cultural history of *Bacillus subtilis* fermentation is well known, research on modern uses and consumption of *Bacillus subtilis* is comparatively very recent. Clinical trials have shown that *Bacillus subtilis* is safe for consumption, and beneficial for digestive health [3, 4].

The term *probiotic* means "for life" in Greek. It was first used in 1965 by Lilly and Stillwell [3] to name microorganisms that are beneficial to consume. The general health benefits of consuming probiotics have been shown in both animal and human studies. As a component of the human microbiome, *Bacillus subtilis* has the ability to promote gastrointestinal health, including helping its host in digestion, making it an ideal probiotic.

The purpose of this double-blind, randomized study was to determine the efficacy of *Bacillus subtilis* DE111 in capsule form for regulation of bowel movements. 50 adults (18-65 years of age at the time of participation) suffering from occasional constipation and/or diarrhea were assigned to consume either 1 x 10^9 CFU of DE111 or placebo. Each group was instructed to consume one capsule per day with a meal for 90 days. Efficacy was assessed through participant-reported bowel movement (BM) records as well as dietary intake logs.

II. MATERIALS AND METHODS

Composition of Supplement and Placebo

Each DE111 supplement contained a dose of 1 x 10^9 CFU of *Bacillus subtilis* DE111 diluted to concentration with low moisture rice dextrin. Placebo supplements contained only low moisture rice dextrin. Both DE111 and placebo were encapsulated in size one opaque vegetable capsules.

Subject Recruitment

Participant recruitment included online postings to Clinical Connection, Atlanta Job Exchange, and social media for local interest sites. Furthermore, recruitment flyers were posted throughout the metro Atlanta area.

Participant Demographics and Inclusion Criteria

This study aimed to use a diverse participant population representative of the general population. 50 adults, ages 18-65 at time of participation and suffering from occasional constipation and/or diarrhea completed this study (Initially, 65 individuals were enrolled, 7 were dropped due to their initial bloodwork results, 6

were dropped due to CRP levels higher than 5 mg/L and 2 opted out mid study). Occasional constipation/diarrhea was defined as frequency ranging from 1-5 episodes per month, with each episode lasting a minimum of 24 hours. Medical history questionnaires, digestive health questionnaires, and initial blood samples were collected and assessed by a board-certified physician to determine general health of participants before complete inclusion. Blood samples were also collected at days 45 and 105, and health questionnaires were completed on days 1, 15, 45, 75, and 105. Blood samples were collected at LabCorp locations that were convenient to each participant. C-reactive protein (CRP), lipid panels, and complete metabolic panels (CMP) levels were used as a safety screen to determine participant health at time of inclusion. Trained phlebotomists used routine venipuncture procedures to collect blood samples from participants.

Data collection procedures

Participants recorded daily entries of their dietary intake and BM records throughout the 90 days. The daily BM logs required participants to score their bowel movements using the Bristol Stool chart. Additionally, digestive health questionnaires were completed on days 1, 15, 45, 75 and 105 using the online survey service Survey Monkey. The questionnaires required participants to rate their general digestive health by the following scale: 0= symptom is not present; 1= mild/sometimes; 2= moderate/often; and 3= severe/almost always. Participants had the option to use controlled electronic documents or hard copy packets to complete the daily BM logs and digestive health questionnaires.

Incentives, Follow-ups, Compensation

Participants could withdraw from the study at any time without penalty. Daily interaction encouraged participants to maintain daily tasks, ask questions, and voice concerns. Communication included email and phone calls to remind participants of upcoming questionnaires, and sample collections. Participants who completed the study were paid an honorarium in appreciation for their time.

Table 1. Participant Characteristics

Demographic	Statistic	Probiotic (A) (N=24)	Placebo (B) (N=26)	Females (N=36)	Males (N=14)	Under 30 (N=28)	Over 30 (N=22)	Overall (N=50)
Age	Mean	30.1	32.9	31.5	31.9	23.3	42.6	31.6
	Min-Max	19-53	22-64	19-64	20-53	19-29	30-64	19-64
Bristol Type	Mean	3.48	3.36	3.44	3.37	3.45	3.37	3.42
	Min-Max	1-7	1-7	1-7	1-7	1-7	1-7	1-7
*Transit health	Mean	3.29	3.12	3.20	3.18	3.23	3.15	3.20
	Min-Max	1-4	1-4	1-4	1-4	1-4	1-4	1-4

*BM health was scaled from the self-reported Bristol Stool types: 1 = Very Poor**;** 2 = Poor; 3 = Fair; 4 = Good

Statistical Analysis

The participants were tested for differences between the DE111 and the Placebo groups as well as differences within groups. The subgroups included gender female vs male and age, under 30 (n=28) and over 30 (n=22), to preserve a larger sample size and balance proportion between groups (see Table 1).

The Bristol stool types describe various states of bowel transit health from hard to pass stools, constipated (Bristol 1-2); normal (Bristol 3-5); and very loose stools, diarrhea (Bristol 6 and 7). This scale was regrouped into a ranking scale starting from 1 (Bristol 1 and 7; the worst scale numbers for diarrhea and constipation), to 2 (Bristol 2 and 6), 3 (Bristol 5) and ideal type 4 (Bristol 3 and 4). Collectively these were referred to as "BM Transit Health".

Bristol types were placed into a binary categorical group consisting of "Normal" (Bristol 3, 4 and 5) and "Non-normal" stools (Bristol 1, 2, 6, and 7). The data on BM state was divided into six 15 day intervals. Interval 1 (days 1-15), Interval 2 (days 16-30), Interval 3 (days 31-45), Interval 4 (days 46-60), Interval 5 (days 61-75) and Interval 6 (days 76-90). All time groups were tested for independence in proportions using the Chi-Square test statistic.

The proportions between groups and the change in proportions from interval 1 to interval 6 within each group was tested using Chi-squared tests for the BM state variable of "normal" and "non-normal" stools. All the hypotheses-based tests of proportions were two-sided and statistical significance was accepted at the p=0.05 level. No adjustments for multiple comparisons were made. The questionnaires were analyzed using a paired sample T-Test comparing group scores.

Independent t-tests were used for between group differences with respect to capsule type, and paired t-tests were used to assess within group differences with respect to time. Between group differences with respect to capsule type are reported. Independent samples T-tests were used to test for differences between Capsule A and Capsule B at each point of the study, baseline, mid and final. Paired samples t-tests were used to assess the differences between Capsule A at baseline, mid-point and post study; and for Capsule B at each point in the study. P-values below 0.05 were considered significant, and p-values between 0.05-0.10 were considered near significant.

All statistical analysis was completed using the R language and environment for statistical computing (version 3.3.2, R Foundation for Statistical Computing, Vienna, Austria).

III. RESULTS

The mean BM Transit Health was significantly different in days 75-90 (last two weeks) vs the first two weeks among the Capsule A group (p-value = 0.0369) moving from non-healthy stools to healthy stools. There were no strong differences found when other ranges of days were compared in the placebo group (Table 2).

Table 2. Mean BM Transit Health for Days 1-15 vs. Days 75-90

		Day 1-15	Day 75-90	P-value
Capsule A	Mean	3.29	3.45	0.0369
Capsule B	Mean	3.06	3.08	0.7386

The results of the Chi-Square test indicated that over time and by Interval 6, the strength of the difference in proportions of normal vs non-normal stool types increased for the probiotic group (see Figure 2).

Comprehensive metabolic panels and lipid panels stayed within normal reference ranges both in the probiotic and placebo groups with no adverse effects or significant serum level differences. CRP levels remained within normal levels for both probiotic and placebo groups throughout the study.

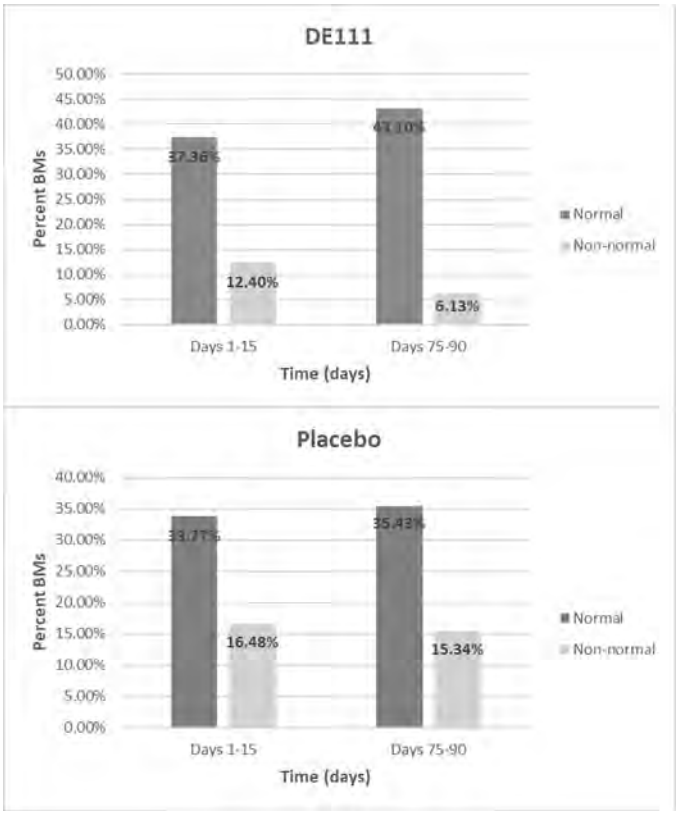
The difference in means between Interval 1 and Interval 6 for the DE111 are statistically significant. By day 90 the proportion of normal stools (43.1%) to non-normal stools (6.13%) in the DE111 group differed significantly (p = 5.866E-08; Chi-squared 29.407) from that in the Placebo group (see Figure 2 and Table 3). The proportion of normal Bristol stools type 3 and 4 increased from 37.36% in week 1 to 43.1% in the last week of the study. The proportions of normal stools in the Placebo group stayed roughly the same from 33.77% to 35.43%, the degree of change was insignificant and not attributable to any other factor in the research (p = 0.137; Chi-squared 2.204). By days 75-90, there is a significant increase in normal stool types of participants in DE111 group. This was not observed in the placebo group which actually showed a decrease in normal stools.

Results of the questionnaires completed were analyzed. Paired sample t-tests of groups showed a reduction in the mean score for the question “Have you experienced alternating constipation and diarrhea?” (0= symptom is not present, 1= sometimes, 2= often, 3= almost always) from a day 1 mean of 0.42 down to a day 15 mean of 0.11 (p = 0.05). No other statistically meaningful differences between the DE111 and Placebo groups or between questions were found.

Table 3. BM State compared by Capsule Group and time and

	Interval	A	B	Group Total	Chi Square	*p-value*
Non-normal	1	76	101	177	4.2511	0.03922
Normal		229	207	436		
Non-normal	2	68	105	173	9.0109	0.002684
Normal		235	207	442		
Non-normal	3	64	85	149	2.6626	0.1027
Normal		237	227	464		
Non-normal	4	70	97	167	3.4943	0.06158
Normal		235	228	463		
Non-normal	5	71	111	182	9.7593	0.001784
Normal		238	210	448		
Non-normal	6	40	100	140	29.407	5.866E-08
Normal		281	231	512		

Figure 2. Total Percentage for Each Participant BM Type.



IV. DISCUSSION

Bacteria make up more than 50% of the composition of a healthy person's stool and play a major role in the quality and frequency of bowel movements. Probiotics are live microorganism that confer a gastrointestinal health benefit to the host. Gastrointestinal regularity may be the result of several potential probiotic mechanisms of action. The presence of probiotics may modify the gastrointestinal microbiota. This beneficial bacterium may release metabolites that can alter gut function, including satiety and motility. Some probiotics can increase the production of lactate and short-chain fatty acids, reducing luminal pH, which has been proposed to enhance gut transit time and reduce inflammation.

DE111 significantly improved gastrointestinal discomfort including constipation and diarrhea over the course of the study. Individuals in the DE111 group reported an increased frequency of normal type stools compared to those in the Placebo group. Therefore, a *Bacillus subtilis* DE111 dose at 1 billion CFU/day may improve occasional constipation and diarrhea while helping to maintain gastrointestinal health.

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9.15 APPENDIX XV- PROBIOTIC (*BACILLUS SUBTILIS*) SUPPLEMENTATION DURING OFFSEASON RESISTANCE TRAINING IMPROVES BODY COMPOSITION IN FEMALE DIVISION I ATHLETES

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BACKGROUND

Interactions between the gut microbiota and host play an important role in the regulation of a multitude of physiological processes. Current evidence suggests that gut-host communication affects cognition [1], epithelial protection, mitochondrial function [2], and may shape metabolic and immune network activity [3-5]. Strenuous physical exertion elicits both localized muscular disruptions as well as systemic physiological stress. Research evidence suggests that high-intensity exercise may be linked to an impaired gut barrier, resulting in endotoxin translocation, pro-inflammatory cytokine production, and impaired nutrient absorption [6-9]. For the athlete, maintenance of this gut barrier is of great interest as gastrointestinal dysfunction and impaired nutrient absorption has been shown to adversely affect acute exercise performance and blunt subsequent training adaptations [10].

Probiotics consist of live microorganisms that employ benefits to their host primarily by supporting the proliferation of beneficial gut microflora [11-13]. Furthermore, probiotics modulate the frequency of the tight junction proteins which regulate the permeability of the intestinal paracellular pathway [14]. By enhancing intestinal barrier function, probiotics serve as preventative agents to defend against adverse effects of pathogens [13,14], promoting positive effects on digestion and immune health [4,15-17]. Additionally, it appears that the beneficial effects of probiotics may be strain-specific, with a majority of probiotic studies investigating *Bifidobacterium* and *Lactobacillus* strains in various special groups (e.g. diabetic, obese) of the general population [11,14,18-20]. It is noteworthy that probiotics of the *Bacillus* strain have been shown to be well tolerated in healthy populations [21], and have garnered attention recently for their potential beneficial effects in a recreationally active population [22]. However, additional human trials evaluating the efficacy of these probiotic strains are needed to provide evidence-based recommendations to patient and clients.

Currently, data on the efficacy of probiotic administration in the athletic population is limited. A bulk of the current literature shows promising effects of probiotics for prevention of acute and chronic illness in endurance athletes during times of intense training [15,23-25]. However, much less is known about the potential benefits probiotics may confer to athletes who regularly engage in resistance exercise. Recently, it has been reported that co-ingestion of a probiotic supplement and protein following muscle damaging exercise resulted in improved perceived recovery, decreased muscle soreness, and tended to decrease markers of muscle damage during 72 hours of recovery [22]. Additionally, it was shown that 21-days of probiotic supplementation attenuated circulating Interleukin 6 concentrations and range of motion decrements in the initial 48 hours following a damaging bout of eccentric resistance exercise [26]. Taken together, it appears that probiotics may have immunomodulatory properties which could aid in the acute regenerative capacity of skeletal muscle repair and functional recovery. Improved acute recovery could potentially allow for increased training capacity in subsequent exercise bouts, leading to enhanced training adaptations.

While studies have evaluated the potential benefit of probiotics on acute recovery from resistance exercise, to date no study has investigated the effects of probiotics on chronic adaptations to resistance training. Thus, the objective of the current study was to determine the effects of *bacillus subtilis* (DE111®) probiotic supplementation on muscle thickness and strength, body composition and athletic performance in Division I female volleyball and soccer athletes.

METHODS

Twenty-three Division I female athletes (19.6±1.0y, 67.5±7.4kg, 170.6±6.8cm) from the university's volleyball (n=10) and soccer (n=13) teams participated in this double blind, placebo-controlled, randomized study. Following an explanation of all procedures, risks, and benefits, each participant provided their written informed consent prior to participation in this study. The research protocol was approved by the Institutional Review Board of the University prior to participant enrollment. Exclusion criteria included the use of medication or other probiotic supplementation, ergogenic aids, or suffering from any medical, muscular, or metabolic contraindications.

Study Protocol

Participants reported to the Human Performance Lab (HPL) on two separate occasions at the beginning and end of the 10-week training intervention following a 10-hour overnight fast. During these visits the participants were tested for body composition, muscle architecture, and isometric power. In addition, athletes reported to their strength and conditioning coordinator on two separate occasions pre and post training, to measure 1RM for bench, squat, and deadlift along with testing vertical jump and pro-agility.

Body Composition

Air Displacement Plethysmography

Body density was estimated using air displacement plethysmography using the BODPOD® (COSMED, Rome, Italy). Prior to each test, the BODPOD was calibrated according to the manufacturer's instructions using a two-point calibration. Prior to testing, athletes were instructed to wear a sports bra, tight fitting compression shorts, and a swimming cap, as well as to remove all metal, including jewelry and watches. Body mass was measured to the nearest 0.01 kg using the system's calibrated scale. All athletes were instructed to sit in the chamber, breath normally, and to minimize any movement. A minimum of two trials were performed. If measurements were not within 150 ml of each other, a third trial was conducted. Thoracic gas volume was estimated using the BODPOD software, which uses standard prediction equations and has demonstrated no difference compared to measured lung volumes [27].

Bioelectrical Impedance Analysis

Total body water (TBW) was determined using multi-frequency bioelectrical impedance analysis (BIA) using the InBody® 570 Body Composition Analyzer device (Biospace, Inc., Seoul, Korea). Body composition from BIA is obtained from the measures of resistance and reactance when an electrical current travels throughout the body. Prior to each assessment the participants' hands and feet were thoroughly cleaned with InBody® provided tissues. Age, height, and sex were manually entered, while a scale positioned within the device assessed body mass. The participant was then instructed from the software to stand fully erect on the measurement electrodes situated on the platform and to hold hand electrodes, with arms extended, without touching the sides of their body. Participants were asked to refrain from moving or talking until the assessment was completed. It has previously been shown that BIA is a valid measurement tool for determining TBW when compared to a deuterium oxide technique [28].

Three-Compartment Model (3C-W)

The criterion percent body fat (%BF) was estimated using the three compartment-water (3C-W) model described by Siri [29]. The equation includes measurements of body density (from the BODPOD), TBW (from the BIA), and body mass (BM). The equation for %BF is listed below:

$$\%BF = [(2.118/\text{Body density})-(0.78 \times TBW(L)/BM(kg))-1.354] \times 100$$

Muscle Ultrasonography

Non-invasive measurements of muscle thickness (MT) were collected using B-mode ultrasound imaging with a 12 MHz linear probe (General Electric LOGIQ P5, Wauwatosa, WI). Measurements for the rectus femoris (RF) were taken at 50% of the distance from the anterior, inferior suprailliac spine to the most proximal point of the patella [30]. Vastus lateralis (VL) measurements were taken in the same fashion as previously stated; however, the sampling location is determined by 50% the straight-line distance between the greater trochanter and the lateral epicondyle of the femur [31]. Prior to image collection, participants laid supine for 5 minutes and the probe was coated with a water-based conduction gel [32]. For measurements of MT, the probe was oriented longitudinally in the sagittal plane parallel to the muscle tissue without depressing the skin. Once images were collected, analysis was completed using Image J software (version 1.45s; National Institutes of Health, Bethesda, MD, USA). MT was determined from the still image as the distance between the inferior border of the superficial aponeurosis and the superior border of the deep aponeurosis. Intraclass correlation coefficients ($ICC_{3,k}$) and standard error of measurements (SEM) for the ultrasound technician were calculated for the RF MT ($ICC_{3,k}$ = 0.99, $SEM_{3,k}$ = 0.02, MD = 0.07 cm) and VL MT ($ICC_{3,k}$ = 0.99, $SEM_{3,k}$ = 0.05, MD = 0.14 cm) from analysis of 10 individuals separated by 24 hours.

Dynamic Strength Testing

One-repetition maximum (1RM) strength was assessed in the bench press, squat, and dead lift exercises. All 1RM testing was performed using methods previously described [33]. Prior to testing, each athlete completed a general warm-up led by the strength and conditioning coach, which included jogging and a dynamic warm-up. Each athlete performed two warm-up sets using a resistance of approximately 40-60% and 60-80% of her perceived maximum, respectively. For each exercise 3-4 subsequent trials were performed to determine the 1-RM. A 3-5 min rest period was provided between each trial. Trials not meeting the range of motion criteria for each exercise or where proper technique was compromised were discarded.

Isometric Strength Testing

The isometric mid-thigh pulls (IMTP) test was utilized to assess peak force (PF) and rate of force development from 0-250ms (RFD250ms). The mid-thigh position was determined for each participant before testing by marking the midpoint distance between the knee and hip joints. Each participant was instructed to assume their preferred deadlift position by self-selecting their hip and knee angles. The height of the barbell was then adjusted up or down to make sure it is in contact with the mid-thigh. An overhand grip with lifting straps was used to ensure grip strength did not limit their capacity to pull maximally. The participants were instructed to pull upwards on the barbell as hard and as fast as possible and to continue their maximal effort for 6-seconds. The force-time curve for each trial is recorded by a force plate (PASCO, Roseville, CA) with a sample rate of 1,000 Hz similar to previous studies [34,35]. PF was defined as the highest force achieved during the 6-seconds isometric test minus the participant's body weight in Newtons. RFD was then calculated with the following equation: RFD = ΔForce / ΔTime. The RFD equation was applied to the predetermined time band 0-250ms which is in accordance with previous studies demonstrating high reliability [35,36].

Performance Testing

A vertical jump testing station (Uesaka Sport, Colorado Springs, CO) was used to assess vertical jump height (± 1. 27 cm). Prior to the test, each athlete's standing vertical reach height was determined by

colored squares located along the vertical neck of the device. These squares correspond with similarly colored markings on each horizontal tab, which indicate the vertical distance from the associated square. Vertical jump height was determined by the indicated distance on the highest tab reached following three maximal, countermovement jump attempts performed from a standing position with feet shoulder width apart.

For the pro-agility test, three cones were placed parallel, five meters apart. The athletes set up for the test in a straddle position facing the middle cone. On their ready, the athletes were instructed to pivot to their right and accelerate as quickly as possible to a cone 5m away and then upon touching the first cone, pivot again to their left and sprint the 10m distance to the furthest cone. Upon touching this cone, the athletes once again pivoted to the right to return to the middle cone as quickly as possible. During each change in direction, the athletes were asked to touch the ground next to the cone. Trials where the athlete failed to touch the ground were discarded. Athletes were allowed three attempts and the fastest time measured in seconds was recorded.

Supplementation Protocol

Participants were required to consume a probiotic (DE111) or placebo (PL) once a day for 10 weeks. The probiotic supplement consisted of 5 billion colony forming units (CFU) *Bacillis Subtilis,* (DE111®, Deerland Enzymes, Kennesaw, GA). On training days, supplementation occurred immediately post-workout with a protein and carbohydrate recovery drink (Gatorade Recover, Gatorade Co., Chicago, IL) consisting of 45g of carbohydrates, 20g of protein, and 2g of fat. This recovery drink was chosen to maximize postprandial muscle protein synthesis [37] and to remain within NCAA macronutrient guidelines for nutritional support. On weekend or non-training days, athletes were required to consume their supplement with a normal meal.

Dietary Logs

During the training and supplement intervention participants were asked to complete a three-day food log (two weekdays, one weekend day) on two separate weeks. Dietary recalls were used to provide an estimate of total kilocalorie intake (kcal) and macronutrient distributions (carbohydrate, protein, and fat) of the athlete's typical weekly diet. All dietary analysis was completed using the MyFitnessPal application (Under Armour Inc., Baltimore, MA), which contains a large, detailed US-branded food database.

Offseason Training Protocol

All athletes completed the same periodized (traditional linear) resistance training program for 10 weeks (3 days · week^{-1}). The program incorporated upper- and lower-body workouts centered on three core lifts (bench press, squats, and dead lifts) and commonly referred to as the 'Wendler 5/3/1" [38]. This program organizes progressions on each core exercise over 4-week segments (i.e., 1 week of 3 sets of 5 repetitions, followed by 1 week of 3 sets × 3 repetitions, followed by 1 week of 1 x 5/3/1 repetitions, and then finally a lighter "unloading" week of 3 sets ×5 repetitions). Accessory lifts followed a higher volume pattern (i.e. 3-4 sets, 8-12 repetitions). In addition to strength training, the athletes participated in team conditioning, agility, jumping, and sprint work (3 sessions · week^{-1}). These workouts consisted of approximately 30-40 minutes of sport-specific skill development and conditioning-related work. All training sessions were performed under the supervision of a certified strength and conditioning specialist.

Statistical Analysis

Statistical evaluation of performance, anthropometric, and subjective data was be accomplished using separate two-way (group x time) repeated measures analysis of variance (RMANOVA). Prior to the RMANOVA, all data were assessed for normal distribution, homogeneity of variance, and sample independence. When a significant group × time interaction was observed, independent samples t-tests were performed for each dependent variable at each testing session between groups; dependent samples t tests within each group were performed; and delta scores were calculated and independent samples t tests between groups were performed. Group differences were further assessed via effect sizes (η^2_p; partial eta squared). Effect sizes were interpreted as small (0.01 – 0.059), medium (0.06 – 0.139), or large (> 0.14) as previously recommended [39]. An alpha level was set at $p \leq 0.05$, and all analyses were performed using SPSS version 24.0 (SPSS, Inc., Chicago, IL).

RESULTS

Following 10-weeks of resistance training, significant main effects for time ($p<0.001$) were observed for squat 1RM, deadlift 1RM, bench press 1RM, and vertical jump. However, there was no main effect for time for pro-agility, IMTP PF, or IMTP RFD250ms. Additionally, no significant group x time interactions were observed for any measure of strength or athletic performance. All measures of strength, performance, and body composition before and after 10-weeks of off-season resistance training are presented in Table 1.

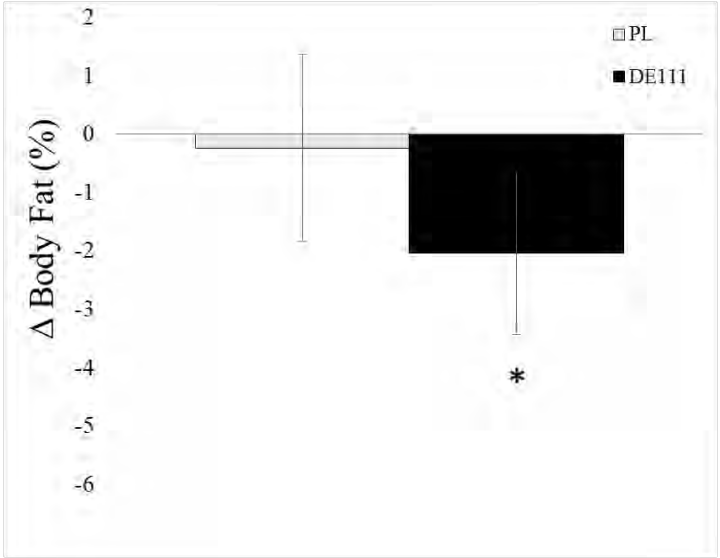
Table 1. Strength, Performance, and Body Composition Changes Following 10-weeks of Offseason Training

Variable	Group	Pre	Post	Time	Time x Group
Strength Measures					
Squat 1RM (kg)	DE111	73.3 ± 11.2	87.1 ± 12.6	p<0.000	p=0.394; n^2=0.043
	PL	74.1 ± 15.3	93.4 ± 19.0		
Deadlift 1RM (kg)	DE111	85.0 ± 14.5	96.0 ± 11.2	p<0.000	p=0.343; n^2=0.056
	PL	81.8 ± 13.1	90.6 ± 16.4		
Bench Press 1RM (kg)	DE111	45.3 ± 8.0	48.0 ± 8.5	p<0.000	p=0.633; n^2=0.012
	PL	42.8 ± 5.3	46.9 ± 6.3		
Performance Measures					
Vertical Jump (cm)	DE111	50.8 ± 5.9	53.3 ± 6.1	p<0.000	p=0.405; n^2=0.041
	PL	54.2 ± 7.8	56.0 ± 8.4		
Pro-Agility (sec)	DE111	5.07 ± 0.23	5.11 ± 0.21	p=0.070	p=0.794; n^2=0.004
	PL	4.97 ± 0.17	5.04 ± 0.19		
IMTP PF (N)	DE111	1570.3 ± 303.7	1598.1 ± 282.6	p=0.150	p=0.351; n^2=0.049
	PL	1334.3 ± 208.7	1446.9 ± 221.5		
IMTP RFD 250ms (N)	DE111	3450.5 ± 1833.0	3336.0 ± 1676.5	p=0.923	

	PL	2740.9 ± 1340.5	2794.3 ± 1311.9		p=0.761; n^2=0.005
Body Composition Measures					
Body Mass (kg)	DE111	70.0 ± 8.4	69.7 ± 7.6	p=0.171	p=0.055; n^2=0.181
	PL	66.6 ± 5.1	68.2 ± 5.4		
Body Fat (%)	DE111	25.1 ± 3.98	23.0 ± 2.94	p<0.000	p=0.015; n^2=0.289
	PL	21.0 ± 5.36	20.0 ± 5.25		
Rectus Femoris Thickness (cm)	DE111	2.22 ± 0.29	2.29 ± 0.27	p=0.015	p=0.500; n^2=0.024
	PL	1.98 ± 0.32	2.09 ± 0.28		
Vastus Lateralis Thickness (cm)	DE111	1.75 ± 0.31	1.71 ± 0.22	p=0.623	p=0.082; n^2=0.151
	PL	1.42 ± 0.28	1.49 ± 0.23		

No significant (p>0.05) main effect for time or interaction was observed for body mass. A significant main effect for time (p<0.05) and a significant group x time interaction (p=0.015) was observed for BF%. Delta BF% scores (POST – PRE-values) further indicated that the DE111 group experienced greater decrease in BF % (-2.05±1.38%) compared to PL (-0.2±1.6%; Figure 1). A significant main effect was observed for RF thickness (p=0.015) with both groups experiencing an increase in muscle thickness compared to pre-values. However, only 57% of the participants experiences an increase in muscle thickness which exceeded the minimal difference (0.07cm) calculated in our ICCs. Additionally, no main effect for time was observed for VL muscle thickness and no interactions were seen for RF nor VL thickness between treatment groups.

Figure 1. Changes in body fat percentage following 10-weeks of training. *=significantly greater change compared to placebo (PL).



No significant differences in average daily caloric intake were observed between the DE111 (1836.4kcals) and PL (1804.1kcals) groups. In addition, no significant differences were seen between groups in

carbohydrate (DE111:38.4g vs. PL: 215.1g), protein (DE111: 91.0g vs. PL: 94.5g) and fat (DE111: 60.5g vs. PL: 63.1g) intakes. Furthermore, both DE111 and PL supplements were well tolerated, and no adverse side effects were reported.

DISCUSSION

The major finding of this study was that probiotic supplementation with DE111 resulted in superior improvements in body composition following 10-weeks of resistance training compared to a placebo. Furthermore, our data showed that 10-weeks of offseason training resulted in significant improvements in 1RM strength (bench press, squat, deadlift) and vertical jump height with DE111 supplementation providing no additional benefit compared to placebo. Additionally, we observed no difference between groups in pro agility time, IMTP PF, IMTP RFD, and muscle thickness. To the best of our knowledge, this is the first study to investigate the effects of probiotic supplementation on resistance training induced adaptations.

Following 10-weeks of training, both groups experienced improvements in body fat percentage similar to values previously reported in female collegiate basketball players following eight-weeks of offseason resistance training and protein supplementation [40,41]. Our findings also revealed greater reductions in BF% in the probiotic group (-2.1%) compared to placebo (-0.2%). Currently, there is a significant gap in the literature with regards to probiotics and body composition in healthy adults. However, a growing body of evidence suggests that in overweight and obese individuals, modulation of the gut microbiota produces favorable reductions in body fat mass [16,18,42,43]. In healthy, normal weight adults, probiotic supplementation has been reported to attenuate increases in body fat mass during a prolonged high-fat diet [19]. Furthermore, it has been observed that just three-days of a hypercaloric diet (3400kcal) has the capacity to alter the gut microbiome, resulting in an additional energy harvest of 150kcals in lean and obese individuals [44]. Taken together, while the participants in our study on average did not report high average daily caloric or fat intake, it is possible that the probiotic supplement reduced energy storage following potential episodic over-feedings during the 10-weeks. An increase in weight as little as 2% of body mass has been previously shown to impair vertical jump and sprinting performance [45]. Additionally, improvements in body composition have reported to be modest over multiple training seasons in female athletes [46], and accumulation of fat mass is often experienced in the offseason [47]. Therefore, the findings of the present study may prove useful to athletes seeking to alter body composition in pursuit of improved performance, as well as those in weight restricted or aesthetic competitions.

It is important to note that while the underlying mechanisms of probiotic induced improvements in body composition were outside the scope off this investigation, evidence suggests that gut microbiota composition has wide reaching effects on the human body [2,3]. These microorganisms beneficially modulate intestinal permeability, which may play a role in the absorption of protein post workout after acute muscle breakdown. It has been previously reported that high-intensity interval training and resistance exercises increase markers of intestinal damage [7,8] and may impair dietary protein digestion and absorption during post exercise recovery [6]. This impairment in absorption may lead to a reduced capacity for amino acid uptake and may blunt training adaptations. In the present study, increased protein absorption in the probiotic group may have contributed to the improvements in body composition by increased dietary protein induced thermogenesis [48] and altered satiety signaling [49]. On average, our athletes had a daily consumption of 1.6g $\cdot$ kg^{-1} of protein including the provided post-workout nutrition (20g protein). While the supplemental protein allowed these athletes to meet recommended range of protein intake for supporting lean muscle accretion (1.4-2.0 g $\cdot$ kg^{-1} $\cdot$ d^{-1}), intakes above this reference

range have been suggested for additional improvements in body composition [50]. Thus, improved amino acid uptake in the probiotic group may have allowed for more efficient protein digestion, simulating the effects of a higher daily protein intake. Nevertheless, future work is needed to investigate potential underlying mechanisms for the observed improvements in body composition.

Based on a previous study utilizing a probiotic of the *bacillus* strain [51], we speculated that probiotic supplementation would promote improved dietary protein absorption and utilization, resulting in enhanced muscular adaptations following training. Although previous literature examining acute protein absorption highlight the relevancy of probiotic supplementation [6,51], it appears that co-administration of protein and probiotics do not augment the increase in RF or VL muscle size from 10-weeks of resistance training in trained athletes who habitually consumed adequate dietary protein. There was a significant time effect for an increase in RF muscle thickness, with no significant increase seen in VL muscle thickness. However, due to the number of participants which did not exceed the RF minimal difference (43%), these data may have little practical importance. These findings are in agreement with a previous investigation reporting no change in VL thickness following 14-weeks of a periodized resistance training program in Division I softball players [52]. Conversely, previous work has shown improvements in both RF and VL thickness following strength training programs of various lengths [30,53,54]. Dietary recalls revealed the athletes' overall caloric intake was about 400-600 calories below what would be predicted for an active female population [55]. Thus, while the athletes were able to meet protein recommendations, overall caloric intake may not have been sufficient to observe substantial hypertrophy. Further studies examining various daily caloric and protein intakes, protein types (i.e. soy, pea, casein), and longer training periods are needed to advance our understanding of the potential benefits of probiotics on muscular adaptations.

All participants experienced improvements in 1RM strength measurements following training, with no differences observed between experimental groups. These data are in agreement with previous investigations reporting similar strength adaptations following offseason resistance training [40,41,52]. However, no improvements in IMTP PF or IMTP RFD were observed. Though various studies have investigated the relationship between IMTP and athletic performance [34,35], only one study has reported improvements in IMTP performance following chronic resistance training [36]. It is possible that 10-weeks is not a sufficient training duration to see improvements in IMTP performance in trained collegiate athletes. Additionally, the athletes in our study did not experience improved pro-agility times in either group following offseason training. This is in contrast to a previous study reporting significant improvements in agility times following offseason training in female collegiate volleyball [56] and basketball players [40,41]. While our participants were comprised from two separate athletic teams (i.e. volleyball and soccer) and completed matching resistance training programming, sport specific team training and agility sessions were not controlled in this study. As soccer and volleyball require unique skills for sport success, team-specific activities were not the same for all participants over the 10-week intervention. Thus, differences in sport specific training may explain why we did not observe a training effect for agility performance.

Conclusion

In summary, we report for the first time that supplementation with the probiotic DE111 may improve body composition in female collegiate athletes in conjunction with offseason resistance training. These data are of interest to a wide array of athletes attempting to optimize body composition changes in the offseason. Additionally, as acute and chronic resistance training induced stressors have the potential to negatively impact immune, neuroendocrine, and gut health, promoting an optimal microbiota could benefit athletes.

Nevertheless, further research is needed to investigate the potential benefits of probiotics in relation to protein absorption, acute exercise recovery, body composition, and training induced muscular adaptation in athletes.

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Availability of data and materials

All data is presented in the main paper.

Authors Contributions

Experimental Design: JCT, JRT, SBJ, AMT, MDR, TAV, YF, GTM. Data Acquisition: JCT, JRT, SBJ, AMT WCV, DB, CCC, KLS. Data Interpretation: JCT, JRT, SBJ, AMT, WCV, DB, CCC, KLS, MDR, TAV, YF, GTM. Manuscript Writing: JCT, JRT, TAV, YF, GTM. All Authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Lipscomb University Institutional Review Board. All participants provided informed consent prior to taking part in the study.

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9.16 APPENDIX XVI- EFFECTS OF PROBIOTIC (*BACILLUS SUBTILIS* DE111) SUPPLEMENTATION ON IMMUNE FUNCTION, HORMONAL STATUS, AND PHYSICAL PERFORMANCE IN DIVISION I BASEBALL PLAYERS

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BACKGROUND

Athletes regularly engage in rigorous exercise training which leads to accumulating amounts of physical stress. While daily moderate intensity physical activity has been shown to have positive effects on the immune system (45), prolonged periods of intense training and competition may lead to immune dysregulation (18, 36, 37). As a result of mucosal and systemic immune suppression, it is common for competitive athletes to become susceptible to infections, which may reduce the frequency and quality of physical training and athletic competition (12). In addition to being venerable to infection, overly fatigued athletes are found to have altered levels of pro and anti-inflammatory cytokines in circulation (15, 24). For instance, elevated circulating TNF-α in elite male rowers was significantly associated to depressed mood, sleep disturbances, and physical stress (38). Moreover, TNF-α acts to impair protein synthesis in skeletal muscle by decreasing mRNA translational efficiency (30). This combination of factors may limit an athlete's ability to properly recover from acute training bouts and may ultimately impair training adaptations.

While athletes are often subjected to excessive levels of physical stress as a byproduct of training demands, other stressors are often overlooked. For instance, collegiate athletes regularly engage in periods of high physical stress accompanied with prolonged travel, academic rigor, and other physiological stressors. College athletes who are under a large amount of physical and academic stress have recently been shown to be more susceptible to sustaining injury during these times of increased strain (39). To counter this, biomarker monitoring is gaining momentum in the athletic realm as a method to detect periods of excessive negative physiological stress (31). Furthermore, it has been suggested that utilizing an assembly of diverse biomarkers may provide the most effective strategy in evaluating intricate balance of anabolic and catabolic processes in athletes (34, 52).

To attenuate the increasing levels of physiological strain associated with training, athletes often implement nutritional strategies to support immune health. Probiotic supplementation, for instance, is a strategy which is receiving considerable attention as a countermeasure for training-induced stressors (47). Probiotics are live organisms that when consumed, impose a wide array of beneficial physiological effects on humans, most notably promoting improved gut microbiota (4). These microorganism have been shown to exert immunomodulatory effects (32) by decreasing pro-inflammatory cytokines in circulation (29) and supporting mucosal defense (17, 42). In athletes, probiotics have been reported to reduce the number, duration, and severity of infections (10, 17, 57). Thus by improving resistance to infection, attenuating low-grade inflammation, and improving nutrient absorption, probiotic supplementation may be a practical strategy to support athlete health and adaptation (9, 47).

While probiotics appear to have a generally positive effect on athlete immune function, studies regarding its efficacy on improving exercise performance are less clear. In endurance athletes, a multi-strain probiotic significantly improved time until fatigue in males running at 80% of their ventilatory threshold (50) whereas others have reported no effect of probiotics on performance (10, 42, 57). Regarding resistance exercise, Jager et al., (26) found that co-ingestion of protein with a *Bacillus* strain probiotic attenuated range of motion decrements in recovery following an intense bout of resistance exercise possibly by improving nutrient absorption (28). Furthermore, 10-weeks of *Bacillus subtilis* supplementation in conjunction with adequate post-workout nutrition was shown to improve body composition in female collegiate athletes (Toohey 2018). Despite recent interest, only a limited number

of investigations have explored the effect of probiotic supplementation on training outcomes in resistance-trained individuals (14, 25) and more data is needed to delineate their effects on performance.

Therefore, the aim of the present study was two-fold. First, we sought to examine the effects of 12-week of daily probiotic supplementation on the immune and hormonal profile in college athletes during a period of increased academic and physical stress. Second, we evaluated the effect daily probiotic supplementation on physical and performance adaptations in Division I collegiate baseball players following 12-weeks of offseason training. With this investigation, we sought address gaps in the literature regarding probiotic supplementation in team sport athletes as well as further explore potential mechanism by which probiotics may improve athlete health and performance.

METHODS

Twenty-five Division I male baseball athletes (20.1±1.5y, 85.5±10.5kg, 184.7±6.3cm) participated in this double blind, placebo-controlled, randomized study. Participants were randomly assigned to a probiotic (PRO; n=13) or placebo (PL; n=12) group. Following an explanation of all procedures, risks, and benefits, each participant provided their written informed consent prior to participation in this study. The research protocol was approved by the Institutional Review Board of the University prior to participant enrollment. Exclusion criteria included the use of probiotic supplementation, ergogenic aids, or suffering from any medical, muscular, or metabolic contraindications.

Study protocol

Participants reported to the Human Performance Lab (HPL) on two separate occasions at the beginning and end of the 12-week training intervention following a 10-hour overnight fast. Additionally, athletes were instructed to report to the lab hydrated while abstaining from caffeine, alcohol, and vigorous exercise for at least 24 h prior to both laboratory testing sessions. During these visits the participants were tested for body composition, muscle thickness, and provided biological samples. Furthermore, athletes reported to their strength and conditioning coordinator on two separate occasions pre and post training, to measure 1RM for squat and deadlift along with testing pro-agility, 10-yd sprint, and standing long jump. Pre-training all 1RM sessions began at the beginning of the fall semester the first week of classes. Post-training, 1RM and performance testing occurred the week prior to final examinations. Since one of our aims was to investigate biomarkers of fatigue and immune function during a stressful period, we chose to conduct our post-training biochemical sample collection during final examination week (39). Additionally, as winter months have been shown to produce additional challenges to the immune system (57), our post-testing biochemical sampling occurred in a winter month as well (December).

Body Composition

Air Displacement Plethysmography

Body density was estimated using air displacement plethysmography using the BODPOD® (COSMED, Rome, Italy). Prior to each test, the BODPOD was calibrated according to the manufacturer's instructions using a two-point calibration. Prior to testing, athletes were instructed to wear tight fitting compression shorts and a swimming cap, as well as to remove all metal, including jewelry and watches. Body mass was measured to the nearest 0.01 kg using the system’s calibrated scale. All athletes were instructed to sit

in the chamber, breath normally, and to minimize any movement. A minimum of two trials were performed. If measurements were not within 150 ml of each other, a third trial was conducted. Thoracic gas volume was estimated using the BODPOD software, which uses standard prediction equations and has demonstrated no difference compared to measured lung volumes (41).

Bioelectrical Impedance Analysis

Total body water (TBW) was determined using multi-frequency bioelectrical impedance analysis (BIA) using the InBody® 570 Body Composition Analyzer device (Biospace, Inc., Seoul, Korea). Body composition from BIA is obtained from the measures of resistance and reactance when an electrical current travels throughout the body. Prior to each assessment the participants' hands and feet were thoroughly cleaned with InBody® provided tissues. Age, height, and sex were manually entered, while a scale positioned within the device assessed body mass. The participant was then instructed from the software to stand fully erect on the measurement electrodes situated on the platform and to hold hand electrodes, with arms extended, without touching the sides of their body. Participants were asked to refrain from moving or talking until the assessment was completed. It has previously been shown that BIA is a valid measurement tool for determining TBW when compared to a deuterium oxide technique (2).

Three-Compartment Model (3C-W)

The criterion percent body fat (%BF) was estimated using the three compartment-water (3C-W) model described by Siri (51). The equation includes measurements of body density (from the BODPOD), TBW (from the BIA), and body mass (BM). The equation for %BF is listed below:

$$\%BF = [(2.118/\text{Body density})-(0.78 \times \text{TBW(L)}/\text{BM(kg)})-1.354] \times 100$$

Muscle Ultrasonography

Non-invasive measurements of muscle thickness (MT) were collected using B-mode ultrasound imaging with a 12 MHz linear probe (General Electric LOGIQ P5, Wauwatosa, WI). Measurements for the rectus femoris (RF) were taken at 50% of the distance from the anterior, inferior suprailliac spine to the most proximal point of the patella (27). Vastus lateralis (VL) measurements were taken in the same fashion as previously stated; however, the sampling location is determined by 50% the straight-line distance between the greater trochanter and the lateral epicondyle of the femur (1). Prior to image collection, participants laid supine for 5 minutes and the probe was coated with a water-based conduction gel (3). For measurements of MT, the probe was oriented longitudinally in the sagittal plane parallel to the muscle tissue without depressing the skin. Once images were collected, analysis was completed using Image J software (version 1.45s; National Institutes of Health, Bethesda, MD, USA). MT was determined from the still image as the distance between the inferior border of the superficial aponeurosis and the superior border of the deep aponeurosis. Intraclass correlation coefficients ($ICC_{3,k}$) and standard error of measurements (SEM) for the ultrasound technician were calculated for the RF MT ($ICC_{3,k}$ = 0.99, $SEM_{3,k}$ = 0.07, MD = 0.19 cm) and VL MT ($ICC_{3,k}$ = 0.99, $SEM_{3,k}$ = 0.01, MD = 0.03 cm) from analysis of 10 individuals separated by 24 hours.

Dynamic Strength Testing

One-repetition maximum (1RM) strength was assessed in squat and dead lift exercises. All 1RM testing was performed using methods previously described (23). Prior to testing, each athlete completed a general warm-up led by the strength and conditioning coach, which included jogging and a dynamic warm-up. Each athlete performed two warm-up sets using a resistance of approximately 40-60% and 60-80% of her perceived maximum, respectively. For each exercise 3-4 subsequent trials were performed to determine the 1-RM. A 3-5 min rest period was provided between each trial. Trials not meeting the range of motion criteria for each exercise or where proper technique was compromised were discarded.

Performance Testing

Ten-yard Sprint

The athletes then completed a standardized general and dynamic warm-up that was consistent with their normal training habits and led by each teams' strength and conditioning coach. A pair of cones and tape affixed to the floor were positioned to denote the "starting line". The athletes were instructed to take their preferred starting stance at the starting line and to begin each maximal trial at their ready. The best of three trials was recorded and used for analysis.

Pro-agility Test

For the pro-agility test, three cones were placed parallel, five meters apart. The athletes set up for the test in a straddle position facing the middle cone. On their ready, the athletes were instructed to pivot to their right and accelerate as quickly as possible to a cone 5m away and then upon touching the first cone, pivot again to their left and sprint the 10m distance to the furthest cone. Upon touching this cone, the athletes once again pivoted to the right to return to the middle cone as quickly as possible. During each change in direction, the athletes were asked to touch the ground next to the cone. Trials where the athlete failed to touch the ground were discarded. Athletes were allowed three attempts and the fastest time measured in seconds was recorded.

Standing Long Jump

Standing long jump performance was assessed using a pre-marked (± 0. 5 in) commercial mat (Sportime, LLC, Norcross, GA, USA). Prior to the test, each athlete stood with both feet placed in the marked "starting area" on the mat. Athletes were instructed to perform a maximal horizontal long jump. Standing long jump distance was determined by furthest distance reached following 3 maximal countermovement jump attempts performed from a standing position with feet shoulder width apart.

Supplementation Protocol

Both the PRO and PL groups completed daily supplementation for 12 weeks. The PRO supplement consisted of 1 billion colony forming units (CFU) *Bacillus subtilis,* (DE111®, Deerland Enzymes, Kennesaw, GA, USA). On training days, supplementation occurred immediately post-workout with a protein and carbohydrate recovery drink (27g protein, 36g carbohydrates, 2g fat) in the presence of a study investigator. On weekend or non-training days, athletes were provided their respective supplements in individual bags and were required to consume their supplement with a normal meal and return the used supplement bags.

Nutritional Analysis

During the training and supplement intervention participants were asked to complete a three-day food log (two weekdays, one weekend day) on weeks one, nine, and 12. Dietary recalls were used to provide an estimate of total kilocalorie intake (kcal) and macronutrient distributions (carbohydrate, protein, and fat) of the athlete's typical weekly diet. All dietary analysis was completed using the MyFitnessPal application (Under Armour Inc., Baltimore, MA, USA), which contains a large, detailed US-branded food database.

Saliva Sampling

Saliva and blood samples were obtained at two time points throughout the study (PRE, POST). All biochemical samples at POST were taken at the same time of day as PRE to avoid potential confounding influence of diurnal variations. Prior to saliva sampling, all athletes rested in a seated position for 5 minutes. With an initial swallow to empty the mouth, unstimulated whole saliva was collected by expectoration into a pre-weighed vial for with eyes open, head tilted slightly forward and making minimal orofacial movement. Study personnel then documented the saliva collection duration and weight of the sample. Saliva flow rate (mL/min) was determined by weighing with saliva density assumed to be 1.0 g/mL (6). After collection, the sample tube was centrifuged at 3000g for 15 min to remove cellular debris and which can negatively impact the accuracy of analysis (48). The supernatant was then aliquoted and stored frozen at -80ºC for later analysis.

Blood Sampling

These blood samples were obtained using a single-use disposable needle with the athlete in a supine position for at least 15 minutes before sampling. All blood samples were collected into two Vacutainer® tubes, one containing no anticlotting agent (6mL) and the second containing K_2EDTA (6mL). The blood in the first tube was centrifuged immediately at 3000g for 15 min while the second tube was allowed to clot at room temperature for 30 min and subsequently centrifuged at 3000g for 15min. The resulting plasma and serum were placed into separately labeled microcentrifuge tubes and frozen at -80°C for later analysis.

Biochemical Analyses

Duplicate saliva samples were analyzed for secretory IgA and IgM concentrations using enzyme-linked immunosorbent assay (ELISA) kits (IgA: Salimetrics, State College, PA, USA; IgM: Abcam, Toronto, ON, Canada). The intra-assay coefficient of variation for saliva IgA was 3.31% and 7.54% for IgM. The IgA and IgM secretion rate were then calculated by multiplying the concentration by the saliva flow rate. Circulating plasma concentrations of TNF-α and serum concentrations of IL-10, zonulin, testosterone, and cortisol were assayed via commercially available ELSIA kits (ALPCO, Salem, NH, USA). To limit interassay variability, all samples for a particular assay were thawed once, and analyzed by the same technician using a FLUOstar Omega spectrophotometer (BMGLabtech, Ortenberg, Germany). All samples were analyzed in duplicate with a mean coefficient of variation of 4.05% for TNF-α, 7.45% for IL-10, 4.10% for zonulin, 4.89% for testosterone, and 3.48% for cortisol.

Offseason Training Protocol

All athletes completed the same triphasic undulating periodized resistance training program for 12 weeks (2-3 days · week^{-1}) (Table 1). Triphasic training is a common periodized resistance training program designed to allow an athlete to eccentrically and isometrically absorb energy before applying it in a dynamic movement (11). This program consists of three mesocyles (3-4 weeks) in which athletes emphasize a particular phase of movement (eccentric, isometric, concentric) while performing their core lifts. In addition to strength training, the athletes participated in team conditioning, agility, jumping, and sprint work (2-3 sessions · week^{-1}). These workouts consisted of approximately 30-40 minutes of sport-specific skill development and conditioning-related work. All training sessions were performed under the supervision of a certified strength and conditioning specialist as well as a certified athletic trainer.

Table 1. 12-week Offseason Resistance Training Program

Phase 1 -Eccentric	**Weeks 1-4**				
Day 1	**Sets x Reps**	**Day 2**	**Sets x Reps**	**Day 3**	**Sets x Reps**
Squat	4x 8-5 w/ :03-:05 ECC	Dead Lift	4x8-5	Hang Clean	4x8-5
Box Jump	4x4	Single Hops	4 x :08seconds	Single Leg Box Jumps	4x5
Mobility	3x10	Single Leg Box Squats	4x5	Inverted Row	4x10
Bench Press	4x 8-5 w/ :03-:05 ECC	Scap Angels	3x10	Single Arm Dumbbell Bench	4x8-6
3 Point Row	4x8 w/ :03-:05 ECC	Dumbbell Incline Press	4x8-4	Exercise Ball Core	4x6
GHD Falls	3x8	Banded Swimmers Row	4x10	Sled Push	4x1
		Banded Face Pull	4x10	Banded Hip Flexor Pull	4x10
		6 Pack Scaps YTA	3x6 :03 ECC		
Circuit 1 50:10x 3	**Circuit 2**	**Circuit 1**	**Circuit 2**	**Circuit 1**	**Circuit 2**
Int/Ext Shoulder Rotation	Split Squat	Airex Floor Touches	Keiser Resisted Lunge	Band Pull-Aparts	Box Step-ups
Plank	TGU	Banded Hip Lifts	Banded X Walks	Keiser SL Twist	Ab Wheel
HK Chops	Pullup	Shoulder Raises	Side Plank Row	Kettle Bell Lunge	Med Ball Slams

Phase 2-Isometric	**Weeks 5-8**		
Day 1	**Sets x Reps**	**Day 2**	**Sets x Reps**
Hang Clean	4x6-4	Dead Lifts	4x6-4
Mobility	3x5	SL Hexagon Hops	4x:08
Squat	4x6-4 w/ :03-:05 ISO	W/Y Negatives	3x8
Lateral Box Jump	4x4	SL Pistol Squat	4x5
DB Incline Bench Press	4x6-4 w/ :03 ISO	Bench Press	4x6-4 w/ :03 ISO
Bear Row	4x8-6 w/ :03 ISO	Battle Rope Variations	3x :30
Sled Push	3x1	Black Burns	3x5
Lateral Lunge	3x8	SL RDL Reaches	3x8
Farmers Carry	3x3	TRX Archor Row	3x8
Pull-ups	2x8, 1x6 w/ :03 ISO	Landmine Rotation and Press	3x8
Standing Keiser Twists	3x10	Med Ball Fielding Drill	3x10
		Exercise Ball Knee Drives	3x10

Phase 3-Concentric	**Weeks 9-12**				
Day 1	**Sets x Reps**	**Day 2**	**Sets x Reps**	**Day 3**	**Sets x Reps**
Squat	4x4-2	Dead Lift	4x4-2	Hang Clean	4x4-2
Box Jump	4x4	lateral Bound	4x6	Dead Bugs	4x5
Mobility	3x5	Inverted Row	3x8	Cross-Over ATYT	3x15
Incline Bench	4x4-3	Bench Press	4x4-3	Mobility	3x10
3 Point Row	4x5-3	Med Ball Chest Pass	4x5	Single Arm Bench	4x4-3
Hip Lift	4x6	BlackBurns	4x5	6 Pack Scaps	4x6
Battle Rope Variations	3x:30	Single Leg Squat	4x5	Lateral Sled Pull	3x1
Inline Board Lunge	3x8	Side Plank Row	3x8	Keiser Single Arm Single Leg Row	3x8
Pull-up	3x8	Band Pull-Aparts	3x10	Med Ball Slams	3x10
Keiser Low Row	3x8	Val Slide Lateral Lunge	3x8	Towel Pull-ups	3x8
Supine Bridge w/ Cross Body Med Ball Throw	3x10	Landmine Touches	3x10	Vertimax Pull Over	3x10

Statistical Analysis

Prior to hypothesis testing, the Shapiro-Wilk test was used to evaluate the assumption of normality for dependent variables. Non-normally distributed data were transformed using the natural log. To identify differences between the experimental conditions on changes in muscle size and strength, an ANCOVA was performed on all measures collected at POST. Associated values collected at PRE were used as the covariate to eliminate the possible influence of initial score variances on the outcomes. Following any significant F-ratio, a paired-samples t-test was used to determine if significant difference existed between measures collected prior to and immediately following 12 weeks of training. Group differences were further assessed via effect sizes (η^2p; partial eta squared). Effect sizes were interpreted as small (0.01 – 0.059), medium (0.06 – 0.139), or large (> 0.14) as previously recommended (19). An alpha level was set at $p \leq 0.05$, and all analyses were performed using SPSS version 24.0 (SPSS, Inc., Chicago, IL).

RESULTS

No significant differences were observed between groups for compliance, with all athletes achieving ≥ 92% with an average compliance of 98.8% across groups. No significant differences in average daily caloric intake were observed between PRO (2404 ± 494.3 kcals) and PL (2369 ± 616.3 kcals) groups. In addition, no significant differences were seen between groups in carbohydrate (PRO: 262.2 ± 52.3 g vs. PL: 251.4 ± 62.6 g), protein (PRO: 122.3 ± 33.3 g vs. PL: 128.0 ± 40.1 g) and fat (PRO: 91.3 ±28.7 g vs. PL: 86.5 ± 24.1 g) intakes. Furthermore, both PRO and PL supplements were well tolerated, and no adverse side effects were reported.

Strength, Performance, and Body Composition

Changes in strength, performance and body composition are presented in Table 2. There were no group differences observed between PRO and PL for any measure of strength, performance or body composition. Collectively, significant improvements ($p < 0.001$) were observed in squat 1RM, deadlift 1RM, pro-agility, and standing long jump as a result of 12-weeks of offseason training while no improvement ($p = 0.312$) in 10-yard sprint time was found. Additionally, both groups experienced significantly increased ($p < 0.001$) RF and VL muscle thickness following training while no improvements were seen following Body Fat % ($p = 0.332$).

Table 2. Strength**,** Performance, and Body Composition Changes Following 12-weeks of Offseason Training

Variable		PRE	Covariate	POST	F	p	η^2	95% Confidence Interval	
								Lower	Upper
Squat 1RM (kg)	PRO	116.8 ± 17.1	124.9	141.8 ± 11.2	.459	.505	.020	139.2	159.4
	PL	133.0 ± 32.0		162.2 ± 40.0				143.6	164.7
Deadlift 1RM (kg)	PRO	139.9 ± 12.2	151.3	169.4 ± 21.0	.375	.547	.019	172.2	188.9
	PL	162.8 ± 40.5		188.0 ± 39.1				168.7	185.2
Standing Long Jump (m)	PRO	2.46 ± 0.17	2.50	2.55 ± 0.21	.046	.833	.003	2.53	2.64
	PL	2.54 ± 0.28		2.64 ± 0.19				2.54	2.66
Pro-Agility (sec)	PRO	4.62 ± 0.17	4.60	4.49 ± 0.22	1.152	.300	.071	4.41	4.55
	PL	4.58 ± 0.20		4.50 ± 0.23				4.46	4.60
10yd Sprint (sec)	PRO	1.99 ± 0.86	1.86	1.69 ± 0.12	.852	.371	.054	1.63	1.77
	PL	1.70 ± 0.11		1.66 ± 0.09				1.57	1.73
Body Fat (%)	PRO	14.7 ± 5.6	14.3	14.9 ± 4.8	2.119	.161	.096	13.7	15.7
	PL	14.0 ± 4.9		13.4 ± 4.8				12.9	14.6
RF Muscle Thickness (cm)	PRO	2.39 ± 0.44	2.44	2.51 ± 0.47	.166	.687	.008	2.49	2.64
	PL	2.50 ± 0.28		2.60 ± 0.29				2.46	2.62
VL Muscle Thickness (cm)	PRO	1.73 ± 0.23	1.79	1.78 ± 0.23	.513	.481	.023	1.81	1.89
	PL	1.86 ± 0.33		1.93 ± 0.33				1.83	1.91

Data presented as mean ± SD.

Biochemical Markers

Changes in biochemical markers are presented in Table 3. TNF-α concentrations were significantly ($F = 5.859$, $p = 0.024$ $\eta^2 = 0.020$) lower in PRO (Δ: -0.25 ± 1.10pg/mL, $p = 0.453$) compared to PL (Δ: +0.36pg/mL, $p = 0.160$). There were no other significant group differences in any other biochemical markers examined. However, a trend ($F = 3.41$, $p = 0.078$, $\eta^2 = 0.134$) for lower cortisol concentrations in PRO (Δ: -76.9 ± 222.1 nmol/L, $p = 0.235$) compared to PL (Δ: +39.6 ± 126.03 nmol/L, $p = 0.300$) was observed at POST. Collectively, significant increases were observed for testosterone ($p = 0.045$), LN IL-10 ($p = 0.048$), SIgA rate ($p = 0.031$), and LN SIgM rate ($p = 0.002$) following 12-weeks of offseason training across groups. No main effects for time were observed in any other biochemical marker.

Table 3. Changes in Biochemical Markers Following 12-weeks of Offseason Training

Variable		PRE	Covariate	POST	F	p	η^2	95% Confidence Interval	
								Lower	Upper
TNF-α (pg/mL)	PRO	2.32 ± 0.93	2.37	2.07 ± 0.76	5.857	.024*	.210	1.69	2.49
	PL	2.42 ± 1.49		2.78 ± 0.95				2.35	3.18
LN IL-10 (pg/mL)	PRO	2.79 ± 0.97	2.95	2.89 ± 1.08	.032	.860	.001	2.89	3.22
	PL	3.12 ± 0.88		3.27 ± 1.02				2.91	3.25
Zonulin (ng/mL)	PRO	10.59 ± 2.11	10.14	10.78 ± 2.23	.010	.921	<0.001	9.68	11.04
	PL	9.67 ± 4.32		9.86 ± 4.27				9.60	11.02
Testosterone (nmol/L)	PRO	15.3 ± 6.59	15.7	15.8 ± 6.50	1.89	.183	0.79	14.8	17.4
	PL	16.2 ± 4.56		17.8 ± 4.46				16.0	18.8
Cortisol (nmol/L)	PRO	656.3 ± 237.7	662.8	579.4 ± 183.2	3.411	.078	.134	488.9	678.0
	PL	669.9 ± 224.1		709.5 ± 247.4				606.6	803.5
T/C Ratio	PRO	.024 ± .009	.025	.030 ± .013	.464	.503	.021	.024	.036
	PL	.025 ± .008		.027 ± .009				.020	.033
Total WBC (x 10^9/L)	PRO	5.97 ± 1.50	5.84	7.08 ± 1.85	.235	.632	.011	5.95	8.21
	PL	5.71 ± 1.31		7.46 ± 2.00				6.28	8.64
SIgA Secretion Rate (μg/min)	PRO	105.2 ± 56.4	123.1	176.6 ± 86.5	1.585	.222	.070	138.6	236.7
	PL	141.1 ± 97.2		156.1 ± 98.3				96.0	194.1
LN SIgM Secretion Rate (μg/min)	PRO	8.11 ± 1.45	8.07	8.84 ± 1.07	.452	.509	.021	8.32	9.30
	PL	8.02 ± 1.40		8.5 5± 1.50				8.10	9.07

Data presented as mean±SD. LN=natural log transformation. * significantly different from PL

DISCUSSION

The objective of this study was to examine the effect of daily probiotic supplementation on strength, performance, body composition and biochemical markers in Division I male college athletes. The results of this study indicate that probiotic supplementation did not provide any additional benefits on strength, performance, and body composition following offseason training compared to PL. Furthermore, it appears that probiotic supplementation appears to promote lower circulating TNF-α in resistance trained males. These findings may provide evidence for probiotic supplementation in an athletic population in conjunction with a sound nutrition and training regimen.

As probiotics have previously been shown to modulate pro- and anti-inflammatory cytokines in the body, it has been suggested that probiotics may support an athlete's general immune health (47). Additionally, intense physical training may cause damage to an athlete's gut barrier, resulting in endotoxin translocation, oxidative stress, and a low-grade pro-inflammatory cytokine response (29, 40, 46, 56). In the present study, we found that 12-weeks of probiotic supplementation attenuated increases in TNF-α which were observed in the placebo group. Similar to our findings, probiotic supplementation reduced circulating TNF-α concentrations in endurance trained men (29) while West et al., (57) found that probiotic supplementation likely decreased the magnitude of TNF-α concentrations following acute VO_2max testing. TNF-α is a potent pro-inflammatory cytokine which is designed to serve an essential role in skeletal muscle remodeling (22, 44). However, pronounced levels of TNF-α have been linked suppressed protein synthesis, disordered sleep, and impaired muscular performance (20, 30, 38). Therefore, while no differences in training outcomes were observed, probiotic supplementation still may foster a more favorable physiological state for recovery and adaptation. It is important to note, we studies athletes which

completed a rigorous offseason training program, were in preparation for examination week, and were entering into the winter months. Thus, we cannot speak to the exact cause of the elevations in circulating TNF-α observed in the placebo group. Nevertheless, during a time period where multiple stressors were present, it appears that probiotic supplementation may alter cytokine production in male collegiate athletes.

While IL-10 concentrations in our study did not differ between groups, significant elevations were seen as a result of the offseason training program. Ibrahim et al., (25) found a significant increase in IL-10 concentrations following 12 weeks of circuit training alone and probiotic supplementation alone while the combination of circuit training and probiotics trended towards a significant elevation post intervention. IL-10 is an anti-inflammatory cytokine which is generally elevated post resistance exercise as a means to suppress inflammation and begin the adaptation process (22, 44). While our participants refrained from strenuous activity prior to reporting to the lab, it is possible the observed modest elevation in IL-10 was a lingering anti-inflammatory response from their previous workout session. Future investigations utilizing additional biochemical sampling time points may provide context to interpret these findings.

Immunoglobulins are a heterogeneous group of antimicrobial proteins which appear as the immune system's first line of defense in the response to an antigen (55). IgA is the principal immunoglobulin involved in host defense and has been shown to be suppressed following intense acute (35, 36, 53) and chronic training (17, 37). Contrary to previous reports in endurance athletes (17) and military cadets (54) we found no effect of probiotics on indicators of mucosal immunity in our athletes. However, this is consistent with a number of studies in endurance athletes which found no differences in SIgA or SIgM between groups following probiotic interventions ranging from 4-12 weeks (7, 10, 57). Additionally, another study found no differences in SIgA protein concentration or secretion rate in 24 male and 6 female professional athletes of various sports (42). It is possible that due to the prolonged repetitive nature of endurance exercise, these athletes experience a larger volume of training-induced stress and are thus more prone to immune suppression than strength and team sport athletes. Team sport athletes likely spend a larger amount of offseason time indoors engaging in resistance training compared endurance athletes (e.g. runners) are constantly exposed to the elements for prolonged periods of time.

Testosterone and cortisol represent a hormonal parameters which provide a snapshot of the current anabolic status of an athlete (31). Traditionally, these two endocrine biomarkers are utilized in male athletes to identity and prevent overtraining (21). No significant differences in testosterone, cortisol, or T:C ratio were observed between groups in this study. Nevertheless, a trend was observed for decreased cortisol concentrations in the probiotic group. This is in agreement with previous work which found no effect of probiotics on cortisol concentrations during a period of intense military training (54). However, one study in a non-athletic population reported lower cortisol responses in participants which received a prebiotic (soluble fiber compounds which enhance the growth of gut microbiota) supplement daily for 3 weeks (49). Thus, there is precedent for gut modulatory substances producing a reduced cortisol response in humans. Furthermore, coupled with the probiotic attenuation of TNF-α in our study, coinciding lower average cortisol levels in the probiotic group may indicate a better homeostatic balance for health, recovery, and physiological adaptations.

Zonulin is a protein which plays a central role in modulating intercellular tight junctions in the intestinal endothelium (13). Of late, this protein has been proposed as a novel circulating marker of intestinal permeability (43). In the present study, we found no differences in plasma zonulin concentrations following our 12-week intervention. Previous work found that 14 weeks of probiotic supplementation

resulted in significantly decreased levels of fecal zonulin, indicating an improvement in intestinal barrier integrity (29). While previous literature has observed changes in circulating zonulin following probiotic interventions, it is possible that fecal measurements of zonulin may have been a more sensitive marker to detect changes in our healthy participants. Some investigations have observed compromised gut permeability in response to an acute exercise stress in trained participants following endurance and interval training (33, 46). Thus, future work should seek to characterize intestinal permeability following acute resistance exercise or a competitive event. Lastly, Clarke et al., (8) found that trained athletes possess a healthier, more diverse guy microbiota. Therefore, in highly trained athletes, it may take longer that 12-weeks to observe notable changes in resting gut function and intestinal permeability when no acute exercise protocol is performed.

It has been proposed probiotic supplementation may improve gastrointestinal function resulting in increased absorption of dietary protein (28) which may contribute to enhanced adaptations over the course of a training intervention. In a mouse model, 6-weeks of *Lactobacillus plantarum* produced augmented strength, muscle mass, and type I muscle fiber number while improving endurance swimming performance (5). In the current study, we observed no differences in any measure of physical performance between groups. Additionally, we found no preferential effects of probiotic supplementation on muscle thickness and body composition. To date, only two manuscripts have investigated the effect of probiotic administration on resistance training adaptations. The first investigation (25) found no ergogenic benefit of a probiotic supplement on muscular strength and power following 12 weeks of circuit-resistance training which is in concert with previous work in endurance athletes reporting no effect of probiotics on performance (10, 16, 42, 57). The second study found no preferential benefit of daily *Bacillus subtilis* (5 billion CFU) supplementation on measures of physical performance following 10-weeks of offseason training in female Division I volleyball and soccer athletes (Toohey 2018). However, Toohey et al., did observe significant improvements in body compositions which mimicked those seen in non-athletic populations. While we utilized the same probiotic strain as the previous study in female athletes (*Bacillus subtilis*), we provided our athletes with a smaller daily dose (1 Billion CFU) of probiotic. Therefore, the apparent discrepancy between our results and existing data to date could result from subtle sex and dosage-dependent differences.

In conclusion, our data indicate that 12-weeks of probiotic supplementation results in attenuated circulating TNF-α concentrations in college athletes following offseason training. College athletes typically undergo periods of elevated stress both physically and mentally which may negatively affect recovery and adaptation. One limitation of our study is that we did not collect any direct measures of stressors (e.g. questionnaires) beyond biochemical markers and detailing the training regimen. Future work should include additional assessments regarding sleep patterns, perception of academic stress, and social influences to provide a better picture of the demands of college student-athletes. Nevertheless, due to the fact that we observed no adverse effects of probiotic supplementation, the findings of the present study provide additional support for the possible benefits of probiotic supplementation in an athletic population. However, as the effects of probiotics may be dose, strain, and sex dependent, further research is needed.

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Conflict of Interest

There is no conflict of interests to report

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9.17 Appendix XVII- Tolerance and Effect of a Probiotic *Bacillus subtilis* DE111 Supplement Delivered in Capsule Form

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1. Introduction

The large intestines are colonized with 500 different species of bacteria [1], [2] with 10^{11}/g colon tissue. Gut commensals, described as probiotics, exhibit various beneficial effects for the host [3]. Probiotics are live microorganisms passing through, or residing in, the human gut with low or no pathogenicity and exhibit beneficial effects for the host [1], [3]–[5]. Probiotic supplementation has shown positive results for relief of various ailments, such as antibiotic-associated diarrhea, constipation, allergies, and diabetes [3], [5]–[9]. Probiotics have also exhibited protective properties by producing inhibitory substances, competitive inhibition of pathogenic bacteria, degrading toxin receptors, and stimulating the immune system[3], [4], [7], [10].

Common probiotics are lactic acid producers such as Lactobacillus, Bifidobacterium, and Streptococcus due to their resistance to gastric acids, bile salts, and pancreatic enzymes [3], [11]. Studies have shown that lactic acid bacteria are effective inhibitors of pathogenic, gram-negative, bacterial colonization (e.g. *Salmonella typhimurium, Clostridium difficile,* and *Escherichia coli*) in vitro [1], [3].

Bacillus subtilis are gram-positive, spore forming, rod-shaped bacteria, which have been used as probiotics, competitive exclusion agents, and prophylactics for human and animal consumption [10]. The purpose of this study is to determine the tolerance and efficacy of daily ingestion of one capsule containing approximately 5 x 10^9 colony forming units (CFU)/capsule of *Bacillus subtilis*. Tolerance is assessed through analysis of blood biomarkers within comprehensive clinical metabolic and liver panels, and immune-reactive C-reactive protein (CRP), a substance that reflects acute stress [12]. Tolerance was also assessed through a pre- and post- capsule consumption gastrointestinal symptom questionnaire. Efficacy was determined through blood biomarkers within comprehensive metabolic and lipid panels, bowel movement records, and pre- and post- capsule consumption fecal analyses.

2. Methods

Forty-one participants (19-42 years of age) were recruited for participation in this study, as approved by the Institutional Review Board (IRB) for the Protection of Human Subjects at the University of Wisconsin-La Crosse. The study was randomized and double-blind with daily oral consumption of supplement (approximately 5 x 10^9 CFU/capsule of probiotic (*Bacillus subtilis* DE111*)* or placebo (maltodextrin) for an average of 20 days (range of 15-23 days) (Table 1). One participant dropped out after two days of pill consumption, reporting loose stools.

Table 1. Participant demographics for probiotic and placebo groups.

Gender	Probiotic Group	Placebo Group	Age (years)
Male	**11**	**7**	**23.6 ± 5.3**
Female	**10**	**13**	**22.5 ± 2.4**
Total	**21**	**20**	**23.0 ± 3.9**

Note: Participant ages ranged from 19-42 years. One participant dropped out after two days of pill consumption

Inclusion criteria consisted of adults capable of understanding study procedures, with no reported illnesses at the time of recruitment, nor use of antibiotics for seven days prior to consent.

All participants completed gastrointestinal questionnaires to determine participant baseline and provide comparison upon study completion. Participants were provided a booklet containing a copy of signed consent, serving size of typical foods, food diary pages, Bristol stool charts and bowel movement recording form. Participants were instructed to utilize the serving size and Bristol stool charts to aid in food intake and bowel movement documentation, respectively.

Routine venipuncture procedures were used to collect 15mL of a 12-hour fasted blood sample at the beginning and at completion of the study. Comprehensive metabolic and lipid panels and C-reactive protein (CRP) were analyzed at Gundersen Health System, La Crosse, WI with a Cobas 6000 (Roche/Hitachi, Indianapolis, IN) automated clinical chemistry and immunoassay system.

Participants collected their first natural bowel movement of the day in a Fisherbrand Commode Specimen Collection System (Thermo Fisher Scientific, Waltham, MA) at baseline and upon completion of the study. Samples were transported from the participant's home to the Health Science Center at the University of Wisconsin La Crosse campus in supplied bags, processed immediately, and stored at -80°C until DNA extraction or plating was executed.

Participants were instructed to consume the assigned capsule once per day, with or without food. If a dose was missed, participants 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. Participants were instructed to complete a daily food-intake record, which was to include alcohol consumption, throughout the course of the study. The probiotic capsules, provided by Deerland Enzymes Inc., Kennesaw, GA, contained approximately 5 x 10^9 CFU/capsule of *Bacillus subtilis* DE111and the placebo capsules contained maltodextrin.

All participants completed the provided gastrointestinal questionnaire to gauge final gastrointestinal symptoms. Participants handed in their completed booklets and were given $100 compensation for participation upon completion of the study. Blood was sampled and analyzed as previously described. Fecal samples were collected and analyzed as previously described.

Statistical analysis included the general linear model procedure with within-subjects factor of time (pre- versus post- capsule consumption) and between-subjects factor of capsule type (probiotic versus placebo control group) was conducted with SPSS Version 21.0 (IBM Corp., Armonk, NY). Main effects of time and time by capsule interactions were considered significant at $p < 0.05$.

Fecal plating efforts were divided between the University of Wisconsin-La Crosse and Kennesaw State University. The samples were serially diluted and 10^{-3}, 10^{-5}, and 10^{-7} dilutions were plated. 1 mL of these two dilutions were spread on separate plates to allow growth of *Bacillus subtilis, Escherichia coli, Lactobacillus acidophilus, Bifidobacterium longum*, and *Candida albicans*.

3. Results

Serum fasting glucose was significantly affected by *Bacillus subtilis* DE111 versus placebo supplementation, with a significant time by capsule interaction (alpha ≤ 0.05; P = 0.012) and a significant decrease in serum glucose in the probiotic group (alpha ≤ 0.05; P = 0.001), but no difference in the placebo group, from pre to post capsule consumption (Figure 1). Triglyceride levels maintained the same within the probiotic group, while the control group displayed a significant increase from pre to post based on a pair T-test (alpha ≤ 0.05; P ≤ 0.042) (Figure 1). The cholesterol levels did not change significantly within the standard deviation of the assay for the probiotic group, but showed a significant increase in the control group (alpha ≤ 0.05; P≤0.025) (Figure 2). There was no significant variation from the normal range of CRP by time or capsule.

Figure 1. Metabolic parameters pre to post capsule consumption. Values are expressed as mean ± standard error of the mean, †: significant difference with respect to time and ††: significant difference with respect to time by capsule type

Group	BUN Pre (8-26 mg/dL)	BUN Post (8-26 mg/dL)	Creatinine Pre (0.6-1.1mg/dL)	Creatinine Post (0.6-1.1 mg/dL)	Protein Pre (6.4-8.3 g/dL)	Protein Post (6.4-8.3 g/dL)	Albumin Pre (3.4-4.8 g/dL)	Albumin Post (3.4-4.8 g/dL)	††Glucose Pre (70-98 mg/dL)	††Glucose Post (70-98 mg/dL
Probiotic	12.8 ± 0.7	13.6 ± 0.8	0.90 ± 0.03	0.89 ± 0.02	7.05 ± 0.06	7.11 ± 0.09	4.59 ± 0.04	4.57 ± 0.06	91.0 ± 1.0	85.9 ± 1.4
Control	12.9 ± 0.8	13.1 ± 1.0	0.80 ± 0.02	0.80 ± 0.03	6.78 ± 0.09	6.87 ± 0.11	4.46 ± 0.06	4.51 ± 0.08	86.2 ± 1.3	85.9 ± 1.0

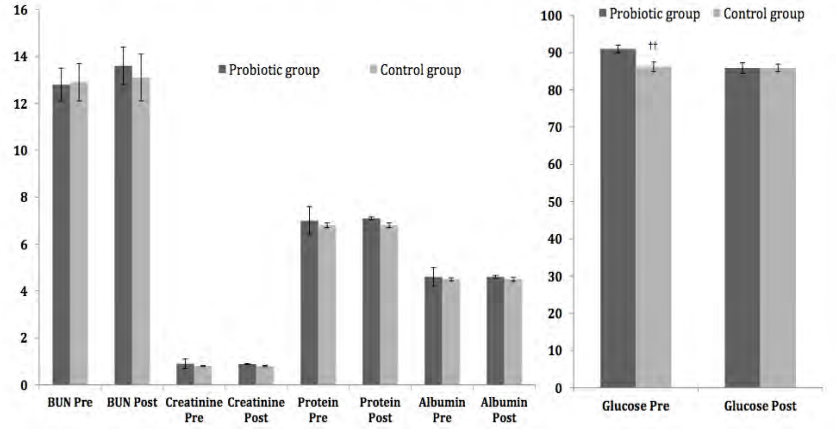
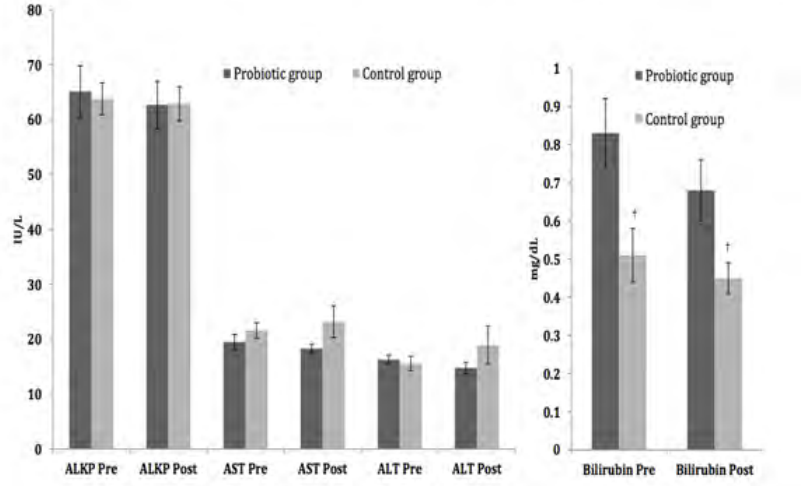


Figure 2. Liver and lipid parameters pre to post capsule consumption. Values are expressed as mean ± standard error of the mean, †: significant difference with respect to time and ††: significant difference with respect to time by capsule type.

Group	†Bilirubin Pre (0.1-1.3 mg/dL)	†Bilirubin Post (0.1-1.3 mg/dL)	ALKP Pre (IU/L)	ALKP Post (IU/L)	AST Pre (0-36 IU/L)	AST Post (0-36 IU/L)	ALT Pre (0-40 IU/L)	ALT Post (0-40 IU/L)
Probiotic	0.83 ± 0.09	0.68 ± 0.08	65.1 ± 4.7	62.7 ± 4.3	19.5 ± 1.4	18.3 ± 0.8	16.3 ± 0.8	14.8 ± 1.0
Control	0.51 ± 0.07	0.45 ± 0.04	63.8 ± 2.9	62.9 ± 3.1	21.6 ± 1.4	23.2 ± 2.9	15.6 ± 1.3	18.9 ± 3.5



While there were no significant differences in gastrointestinal questionnaire answers taken before and after (pre and post) capsule consumption between the probiotic and placebo groups, there were some notable variations between the two groups. Throughout the course of capsule consumption, the probiotic group reported a slight decrease in bothersome nausea and rumbling while the placebo group reported a slight increase in symptoms in these questions. Both groups reported feelings of incomplete bowel movements less often in the questionnaire taken before capsule consumption compared to after capsule consumption.

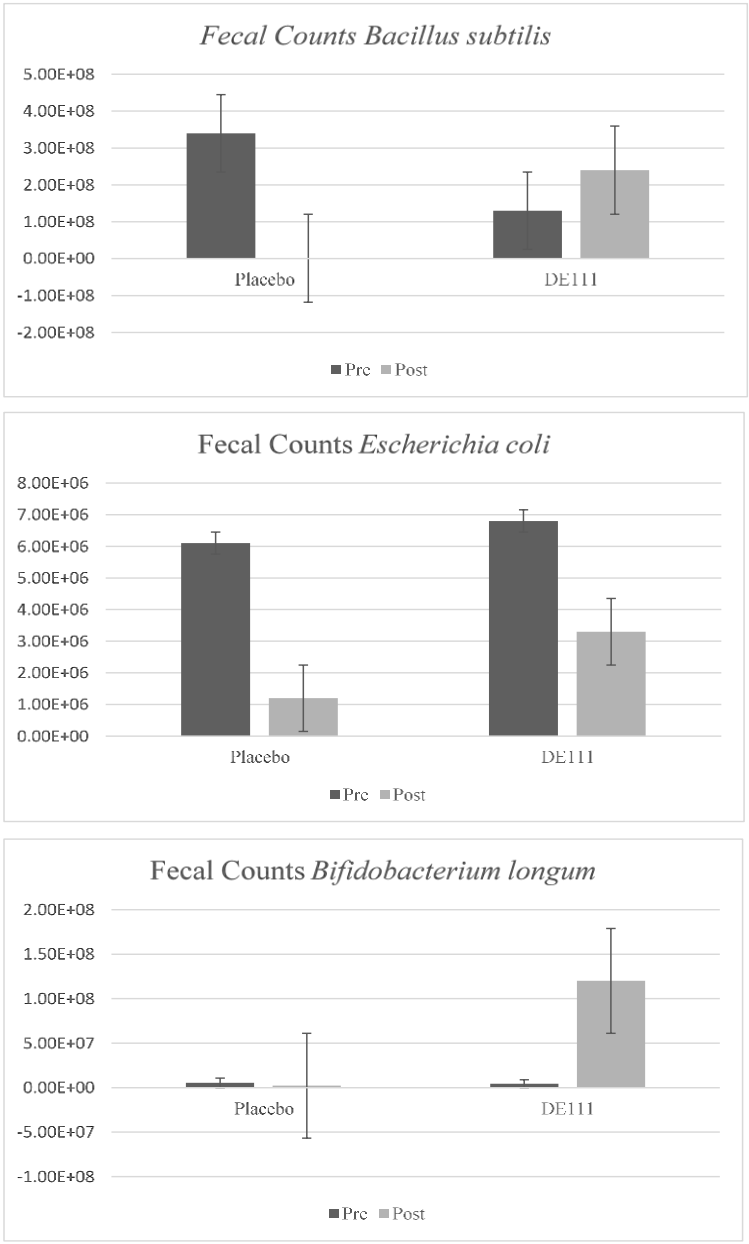
The placebo group had a significant increase in average bowel movements per day when compared to the probiotic group over the course of capsule consumption (alpha ≤ 0.05; $P = 0.015$. Both groups reported feelings of incomplete bowel movements less often in the questionnaire taken before capsule consumption compared to in the same questionnaire taken after capsule consumption.

PCR assays yielded minimal results for the presence of *Bacillus subtilis*, with only four participants' fecal samples positive for *Bacillus subtilis*, all were post-consumption samples within the probiotic group. There was no significant difference between the probiotic and placebo groups pre- to post- capsule consumption. However, there was an upward trend of starting quantity in the probiotic group compared to the placebo group.

There was a significant difference present for *Bacillus subtilis* with respect to time within the probiotic group ($\alpha \leq 0.05$; $P = 0.0053$) and a significant difference between participants factor of capsule type (probiotic versus placebo control group) ($\alpha \leq 0.05$; $P = 0.049$). Participants who were administered the placebo demonstrated a decrease in intestinal levels of the probiotic *Bifidobacterium*, while those who were administered the probiotic experienced a significant increase with respect to time within the probiotic

group (α≤ 0.10; P =0.10) and a significant difference with capsule type (Probiotic versus placebo group) (α≤ 0.10; P =0.08). Subjects who were administered the placebo demonstrated a numerical increase in levels of *Escherichia coli* while those who were administered the Probiotic experienced a slight decrease in *Escherichia coli* (Figure 3). No noticeable differences were observed for either Lactobacillus or yeast.

Figure 3. Fecal plate counts of *Bacillus subtilis, Escherichia coli* and *Bifidobacterium longum* pre and post placebo or DE111 consumption.



4. Discussion

The study population was predominantly a sample of college students, who were willing to provide stool and blood samples, fill out detailed diet and stool records, and complete the GI questionnaire before and after (pre and post) capsule consumption for a $100 honorarium. College student dietary habits are

notoriously irregular, but can be especially so near the end of an academic unit (quarter or semester), when schedules and stress levels change due to final exams. During the time of final exams and before the final sample collections, there was an increase in consumption of alcohol, candy, and fatty foods.

The blood parameters examined were expected to remain the same throughout the course of the study. The only exceptions to this hypothesis were serum glucose and triglycerides. One possibility for the changes observed in serum glucose levels could be from 1- Deoxynojirimycin (DNJ). DNJ is a compound isolated from *Bacillus subtilis* that, when fed to bovine calves, improved diabetic conditions by improving insulin sensitivity [12]. In addition, freeze-dried cultures of *Lactobacillus acidophilus, Bifidobacterium lactis,* and *Lactobacillus rhamnosus* were administered, by gavage twice daily for three days, to male Wistar rats. The delivered probiotics led to reduced blood glucose levels by up to 2-fold in rats with elevated glucose levels.

There was a significant increase in the average number of bowel movements per day within the control group. In addition, no significant difference in either group for bowel movement type was seen. The use of probiotics may alleviate symptoms associated with antibiotic-associated diarrhea, traveler's diarrhea, and symptoms associated with irritable bowel syndrome [13]–[16]. Bowel movement types can be associated with ease of excretion, in addition to efficient elimination of waste material. There was a small, but not significant difference in bowel movement type between the probiotic, averaging a softer, smoother type 4, and control group, averaging a slightly harder, lumpier type 3, throughout the course of the study.

Daily ingestion of one capsule containing approximately 5 x 10^9 colony forming units (CFU)/ capsule of *Bacillus subtilis* 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 of this probiotic on lipid profile in this healthy largely normolipidemic population, there could still be beneficial effects, as demonstrated in some studies, in a hyperlipidemic population. LDL increased in both groups, which may have been a reflection of poor eating habits nearing the end of the semester, but increased less in the probiotic group. Triglycerides levels were maintained in the probiotic group, but increased significantly in the control group. Finally, consumption of *Bacillus subtilis* DE111 in the manner described herein, may improve glucose tolerance, corroborating the findings of non-human animal in vivo and in vitro studies by [6] and [12], respectively. This probiotic is a safe, efficacious dietary supplement for immunity, digestive health, and as a competitive exclusion agent. Daily consumption of the *Bacillus subtilis* DE111 probiotic supplement resulted in a significant effect on gut microflora measured prior to and after capsule consumption in regards to *Bacillus subtilis* and Bifidobacterium.

Declaration of interests' statement

In accordance with Taylor & Francis policy and our ethical obligation as researchers, we am reporting that we have receive funding from Deerland Enzymes, a company that may be affected by the research reported in the enclosed paper. I have disclosed those interests fully to Taylor & Francis, and I have in place an approved plan for managing any potential conflicts arising from this involvement.

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9.18 APPENDIX XVIII- ADHERENCE OF *BACILLUS SUBTILIS* DE111 ON CACO-2 CELLS

Ynés R. Ortega, PhD, MPH
Associate Professor
Center for Food Safety
1109 Experiment St
Griffin, GA 30223

Objective: To determine if *Bacillus* adhere to the surface of Caco---2 cells at various periodsof time.

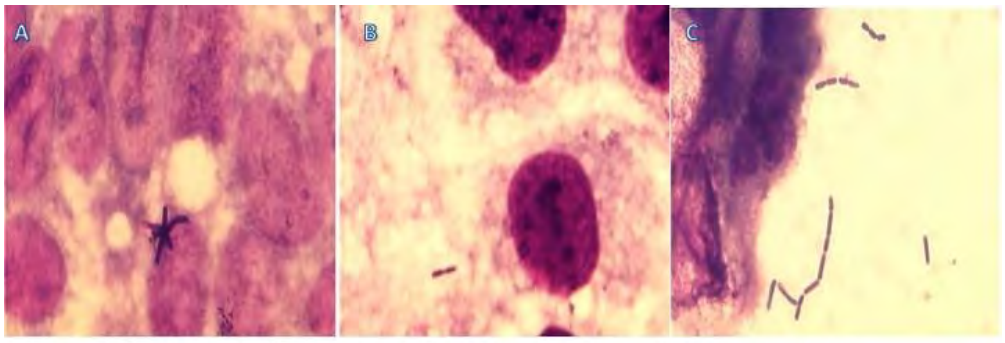
Methods: Caco-2 cells were grown to confluency in multiwall chambers at 37°C and 5% CO_2. *Bacillus subtilis* DE111 was provided by Deerland Probiotics and Enzymes and grown as recommended by the provider (Appendix XI).

One treatment was evaluated in the present study. Bacilli at various concentrations (4, 5, and 6 logs) were exposed to tissue culture media or to pepsine/HCl pH 4 for 5 minutes. After that process, the reaction was neutralized and added to the cells.

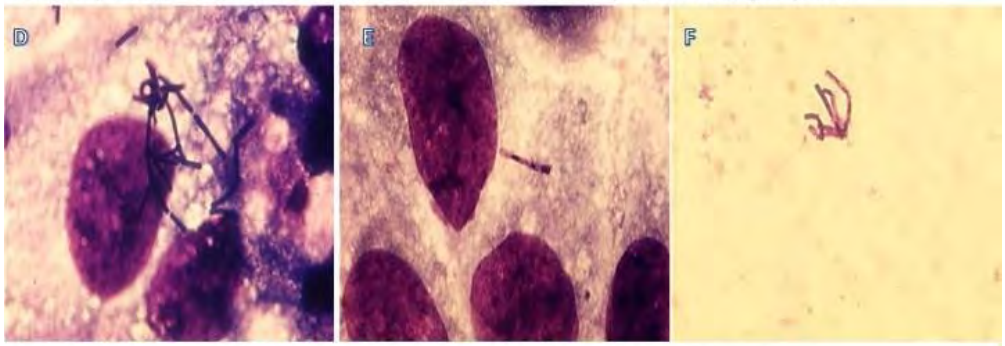
Cells were examined after 30 min, 1 h, and 2 h post exposure. One hundred cells per treatment were counted.

Results: The HCl /pepsine negatively affected the adherence of the bacilli on the surface of the Caco-2 cells. Adherence was more noticeable at 2-hour than at 30 minutes.

No cytotoxicity was noted by microscopy.



30 min post-treatment: A) not treated, B) treated, C) non specific binding to plastic



2 h post-treatment: D) not treated, E) treated, F) non specific binding to plastic

Presence of bacteria on the surface of Caco-2 cells at various periods of time post treatment cells with *Bacillus subtilis* DE111

	No treatment (%)	With treatment (%)
6 LOG	18	0
5 LOG	0	1
4 LOG	0	0
6 LOG	10	2
5 LOG	2	5
4 LOG	0	0
6 LOG	16	4
5 LOG	4	2
4 LOG	1	0

The number of cells per cell increased with time, however *Bacillus* distribution among cells was not homogeneous.

Number of bacteria present in Caco-2 cells with *Bacillus* on them

	Bacillus (N) no treatment	*Bacillus* (N) treatment	range of *Bacillus*/cell no treatment	range of *Bacillus*/cell treatment	average of *Bacillus*/cell no treatment	average of *Bacillus*/cell treatment
30 min						
6 LOG	34	0	1-5	0	1.9	0
5 LOG	0	1	0	1	0	0
4 LOG	0	0	0	0	0	0
60 min						
6 LOG	26	3	2-5	1-2	2.6	1.5
5 LOG	3	9	1-2	1-5	1.5	1.8
4 LOG	0	0		0	0	0
2 H						
6 LOG	100	8	1-26	1-3	6.25	2
5 LOG	9	11	1-4	5-6	2.25	5.5
4 LOG	2	0	2	0	2	0

From:	Catherine Adams
To:	Morissette, Rachel
Subject:	Sending Response to Questions for GRAS Notificaiton DE111 GRN 000831
Date:	Tuesday, April 30, 2019 11:58:33 AM
Attachments:	FINAL FDA GRAS Response 043019.pdf

Attached are the responses to the set of questions regarding GRN 000831 Notification. My thanks for your time in review.

Please let me know if there are questions.

Best regards,
Catherine

Catherine Adams Hutt, PhD, RD, CFS
RdR Solutions
4568 Elm Bottom Circle
Aubrey, TX 76227
and 124 S. Fairfax
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April 30, 2019

Rachel Morissette, Ph.D.
Consumer Safety Officer
FDA Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review

Dear Dr. Morissette,

We appreciate your review of Deerland Probiotics and Enzymes (Deerland)'s GRAS Notice GRN 000831. The following responses are intended to satisfy your questions, and each is addressed below.

Regulatory:
1. Deerland provides the following explanation under Part 4: "Self-Limiting Levels of Use" in the notice:

"The amount offered 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 (no less than 1 mg and no more than 1 gram/dose). This level of use is consistent with dietary exposure and with the safety recognition by regulatory authorities in Japan, Europe, and Canada (EFSA 2007b; Gibson, 2015; NHPD, Health Canada, 2018)."
Please note that under 21 CFR 170.240 "Part 4 of a GRAS notice: Self-limiting levels of use" the regulation is as follows:
In circumstances where the amount of the notified substance that can be added to food is limited because food containing levels of the notified substance above a particular level would become unpalatable or technologically impractical, in Part 4 of your GRAS notice you must include data and information on such self-limiting levels of use.
The explanation provided by Deerland does not adequately address the requirements under 21 CFR 170.240. Please re-state the text to indicate whether there are self-limiting levels of use as defined in the regulation. This includes use levels that would render the food unpalatable or would be technologically impractical to achieve. If self-limiting levels of use exist, please provide data and information to support that. If there are no self-limiting levels of use, please state that.

We retract and revise our submission to state in Section 4, "Self-limiting Levels of Use", to the following:

There are no known self-limiting levels of use.

2. In Part 5 of the notice "Experience Based on Common Use in Food Before 1958," Deerland provides an explanation of the history of use of similar strains in foods throughout the world. However, under 21 CFR 170.245 "Part 5 of a GRAS notice: Experience based on common use in food before 1958" the regulation is as follows: "Bacillus subtilis 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."
While it is possible to have a submission that has a GRAS conclusion based on both scientific procedures and experience based on common use in food before 1958, both bases must be fully supported. Common use in food prior to 1958 has a specific regulatory definition and
If the statutory basis for your conclusion of GRAS status is through experience based on common use in food, in Part 5 of your GRAS notice you must include evidence of a substantial history of consumption of the notified substance for food use by a significant number of consumers prior to January 1, 1958.

In Part 1 of the notice, Deerland states that the basis for its GRAS conclusion is as follows:
should not be confused with history of use. Regarding a common use in foods before 1958 basis, there should be evidence of use prior to 1958 that is reflective of the intended uses proposed. Deerland has not provided information to support a basis of common use in food before 1958 for its intended use of strain B. subtilis DE111. Please either provide a statement that the basis for a GRAS conclusion is by scientific procedures only, or else provide data and information to support that the EXACT intended use in this notice was in common use prior to 1958 in the United States.

This section is not applicable because *Bacillus subtilis* DE111 has been determined to be GRAS by scientific procedures.

3. In Part 1 of the notice, Deerland states that the basis for its GRAS conclusion is as follows:

"Bacillus subtilis 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.
Please revise this statement by removing reference to animals. The CFSAN GRAS Notification program is for intended use in human foods only.

In response to Question 2 above, we state that the basis for GRAS determination is by scientific procedures, thereby ending the paragraph. We remove all remaining in the paragraph, including reference to animals.

Intended Use:

4. In an email dated February 27, 2019, you indicated that the intended use is for conventional foods and non-exempt infant formula for term infants (cow's milk or soy based). However, the notice states on page 16 under Estimated Daily Intake – Infants that "...B. subtilis DE111 is intended to be added to non-exempt term infant formula (including milk-based, soy-based, modified, hydrolyzed, and amino acid-based formula powders and liquids) at levels up to 2 X 108 CFU per 100 mL of infant formula as ready for consumption." Please clarify the intended type of infant formula.

We retract and revise the statement in the email dated February 27, 2019 to the stated intended use is for conventional foods and non-exempt term infant formula.

Microbiology:

5. The notice states that B. subtilis DE111 is deposited in culture collections. Please identify where these culture collections are banked.

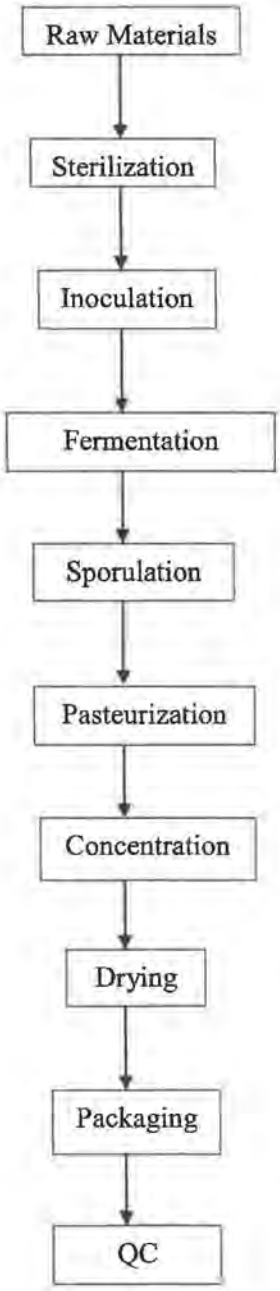
Bacillus subtilis DE111 is available through the culture strain collection of the USDA National Center for Agricultural Utilization Research. Contact is as follows:

Christopher Dunlap, PhD
Research Scientist
National Center for Agricultural Utilization Research
Crop Bioprotection Research Unit
1815 N. University St
Peoria, IL 61604
(309) 681-6339

6. Please confirm that this ingredient is a spore preparation and provide an approximate ratio of spores to vegetative cells.

Bacillus subtilis DE111 is a spore preparation (100%). A pasteurization step is performed at the end of the spore fermentation to kill any vegetative cells that may remain in the culture, prior to downstream processing. No vegetative cells exist in the final product. We have provided a flowchart of our process (Figure 1).

Figure 1. Flow chart of *Bacillus subtilis* DE111 production



7. As this ingredient is intended to be used in infant formula, please consider providing a specification for Cronobacter sakazakii.

Contained in the notice is a microbial specification for conventional food (page 9). We have created a new specification that will be required for infant formula based on the Code of Hygienic Practice for powdered formula for infants and young children (CAC/RCP 66 – 2008).

We add the following to Section 2.3, Specifications for Lots:

Probiotic Count

Test Parameter	**Specification**	**Method**
Total CFU Count	≥200 Million CFU/dose	3.80.308

Physical/Chemical

Test Parameter	**Specification**	**Method**
Color	Cream to dark tan	3.80.181
Texture	Granular powder	3.80.181
Odor	Strong fermentation	3.80.181
Lead	< 1 ppm	ICP-MS
Cadmium	< 1 ppm	ICP-MS
Mercury	< 1 ppm	ICP-MS
Arsenic	< 1 ppm	ICP-MS

Microbiological Standards

Test Parameter	**Specification**	**Method**
Yeast and Mold	≤ 300 CFU/gram	AOAC 20th edition Ch.17 section 17.2.11 p. 26
Coliforms	≤ 100 CFU/gram	AOAC 20th edition Ch.17 section 17.3.04 p. 36
Enterobacteriaceae	≤ 100 CFU/gram	AOAC 20th edition Ch.17 section 17.3.10 p. 50
E. coli	Negative/10 g	USP 41st edition (Vol.5) p. 8158-8164
Staphylococcus aureus	Negative/10 g	USP 41st edition (Vol.5) p. 8158-8164
*Salmonella	Negative/25 g	USFDA. BAM, Chapter 5, *Salmonella, 2007*
**Enterobacter sakazakii* (*Cronobacter* species)	Negative/10 g	BAX® System PCR Assay for *E. sakazakii* (Cronobacter) ISO/TS 22964

*Parameter set as per guidance of the Codex Alimentarius International Food Standards; CAC/RPC 66 208

8. Please provide data from at least three non-consecutive batch analyses to demonstrate that the ingredient can be manufactured to meet the provided specifications.

We have attached three separate DE111 production lots from 2017, 2018 and 2019. Customer specific information has been redacted from these documents; all other information has been retained.

DEERLAND Enzymes | DEERLAND Probiotics

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CERTIFICATE OF ANALYSIS

Product Name:	DE111 100 Billion	**Product Number:**	22102
Release Date:	August 24, 2017	**Lot:**	131451
Expiration Date:	August 2019*		

This is to certify that this lot was manufactured according to FDA's current Good Manufacturing Practices.

Each 1000mg is formulated to contain:

DE111 *Bacillus subtilis* 100 Billion CFU

Test Parameter	Specification	Analysis	Method
pH	6.0-8.0	NA- Powder	3.80.160
Specific Gravity	1.05 g/mL (+/-0.05%)	NA- Powder	3.80.083
Yeast and Mold	≤ 100 CFU/gram	<30 CFU/ gram	AOAC 2014.05
Coliforms	≤ 30 CFU/gram	<30 CFU/ gram	AOAC 991.14
Enterobacteriaceae	≤ 30 CFU/gram	<30 CFU/ gram	AOAC 2003.01
E. Coli	Negative in 10g Sample	Negative in 10g Sample	USP 40 <2022>
Salmonella	Negative in 10g Sample	Negative in 10g Sample	USP 40 <2022>
Staphylococcus aureus	Negative in 10g Sample	Negative in 10g Sample	USP 40 <2022>
Lead	≤ 1 ppm	0.029 ppm	3.80.053** (ICP-MS)
Cadmium	≤ 1 ppm	0.015 ppm	3.80.053** (ICP-MS)
Mercury	≤ 1 ppm	<0.01 ppm	3.80.053 **(ICP-MS)
Arsenic	≤ 1 ppm	0.24 ppm	3.80.053** (ICP-MS)

*Store in an airtight container away from light and moisture at < 25°C. Optimal storage stability is obtained at refrigeration temperatures (4-8°C).
** Results from third party laboratory

QC Released By: Monique Belmudes Digitally signed by Monique Belmudes Date: 2019.04.17 16:44:22 -04'00' **Date:** April 17, 2019

3800 Cobb International Blvd | 2995 Cobb International Blvd | Kennesaw, Georgia 30152

DEERLAND Enzymes | DEERLAND Probiotics

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CERTIFICATE OF ANALYSIS

Product Name:	DE111 100 Billion	**Product Number:**	22102
Release Date:	August 19, 2018	**Lot:**	135550
Expiration Date:	August 2020*		

This is to certify that this lot was manufactured according to FDA's current Good Manufacturing Practices.

Each 1000mg is formulated to contain:

DE111 *Bacillus subtilis* 100 Billion CFU

Test Parameter	Specification	Analysis	Method
pH	6.0-8.0	NA- Powder	3.80.160
Specific Gravity	1.05 g/mL (+/-0.05%)	NA- Powder	3.80.083
Yeast and Mold	≤ 100 CFU/gram	< 30 CFU/gram	AOAC 2014.05
Coliforms	≤ 30 CFU/gram	< 30 CFU/gram	AOAC 991.14
Enterobacteriaceae	≤ 30 CFU/gram	< 30 CFU/gram	AOAC 2003.01
E. Coli	Negative in 10g Sample	Negative in 10g Sample	USP 40 <2022>
Salmonella	Negative in 10g Sample	Negative in 10g Sample	USP 40 <2022>
Staphylococcus aureus	Negative in 10g Sample	Negative in 10g Sample	USP 40 <2022>
Lead	< 1 ppm	0.015 ppm	3.80.053** (ICP-MS)
Cadmium	< 1 ppm	<0.01 ppm	3.80.053** (ICP-MS)
Mercury	< 1 ppm	<0.01 ppm	3.80.053**(ICP-MS)
Arsenic	< 1 ppm	0.20 ppm	3.80.053** (ICP-MS)

*Store in an airtight container away from light and moisture, <25°C. Optimal storage stability is obtained at refrigeration temperatures (4-8°C).
** Results from third party laboratory.

QC Released By: Monique Belmudes Digitally signed by Monique Belmudes Date: 2019.04.17 16:45:29 -04'00' **Date:** April 17, 2019

DEERLAND Enzymes | DEERLAND Probiotics

From concept to commercialization, we add value at every step.

CERTIFICATE OF ANALYSIS

Product Name:	DE111 100 Billion	**Product Number:**	22102
Release Date:	January 23, 2019	**Lot:**	137079
Expiration Date:	January 2021*		

This is to certify that this lot was manufactured according to FDA's current Good Manufacturing Practices.

Each 1000mg is formulated to contain:

DE111 *Bacillus. subtilis* 100 Billion CFU

Test Parameter	Specification	Analysis	Method
pH	6.0-8.0	NA- Powder	3.80.160
Specific Gravity	1.05 g/mL (+/-0.05%)	NA- Powder	3.80.083
Yeast and Mold	≤ 100 CFU/gram	<30 CFU/gram	AOAC 2014.05
Coliforms	≤ 30 CFU/gram	<30 CFU/gram	AOAC 991.14
Enterobacteriaceae	≤ 30 CFU/gram	<30 CFU/gram	AOAC 2003.01
E. Coli	Negative in 10g Sample	Negative in 10g Sample	USP 40 <2022>
Salmonella	Negative in 10g Sample	Negative in 10g Sample	USP 40 <2022>
Staphylococcus aureus	Negative in 10g Sample	Negative in 10g Sample	USP 40 <2022>
Lead	< 1 ppm	<0.01 ppm	3.80.053** (ICP-MS)
Cadmium	< 1 ppm	<0.01 ppm	3.80.053** (ICP-MS)
Mercury	< 1 ppm	<0.01 ppm	3.80.053 **(ICP-MS)
Arsenic	< 1 ppm	0.15 ppm	3.80.053** (ICP-MS)

*Store in an airtight container away from light and moisture, <25°C. Optimal storage stability is obtained at refrigeration temperatures (4-8°C).
** Results from third party laboratory

QC Released By: Monique Belmudes Digitally signed by Monique Belmudes Date: 2019.04.17 16:46:11 -04'00' **Date:** April 17, 2019

9. Please provide the sample sizes used for the negative *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus* specifications provided.

We use as the basis for our microbiological sampling, the U.S. Pharmacopeia National Formulary (2018). <2022> Microbiological Procedures for Absence of Specified Microorganisms- Nutritional and Dietary Supplements. In United States Pharmacopeia [41st Edition (Vol.5), pp. 8158-8164], Rockville, MD.

We weigh out 10 grams of sample for enrichment, as per compendial methods referenced.

10. Please revise the safety narrative in Part 6 to discuss the updated literature that has been published on the safety of B. subtilis since the 1998 example discussed in the notice. Please discuss how these studies pertain to the safety of the intended uses of Deerland's product. An example includes, but is not limited to, the following:

La Jeon et al. 2012. Combined Bacillus licheniformis and Bacillus subtilis infection in a patient with oesophageal perforation. Journal of Medical Microbiology.

Bacillus subtilis appears to have a low degree of virulence to humans. After a thorough literature search, only scarce reports implicating *Bacillus subtilis* in human infections were found. The microorganism does not produce significant quantities of extracellular enzymes or possess other virulence factors that would predispose it to cause infection (Edberg, 1991). According to a statement provided by the EPA on *Bacillus subtilis*, prior to the monographs of Smith in 1946 and 1952; historically, *Bacillus subtilis* was a term given to all aerobic endospore-forming bacilli (EPA, 1997). It is important to note that although earlier literature contains references to infections caused by *Bacillus subtilis*, it is quite possible that many of these infections were associated with *Bacillus cereus*.

Over the past 30 years there have been less than 10 published cases of bacteremia caused by *Bacillus subtilis*. The majority of these cases have occurred in patients with compromised immune status and/or mucosal barrier function due to underlying conditions such as heart disease or other disease state (*i.e.,* periodontitis). Boyle *et al* (2006) stated firmly, "All cases of probiotic bacteremia or fungemia have occurred in patients with underlying immune compromise, chronic disease, or debilitation, and no reports have described sepsis related to probiotic use in otherwise healthy persons." Two case reports have been published on clinical infections in patients consuming probiotics, most commonly strains of *Bacillus subtilis*. However, in both of these cases, the strains isolated from the infection confirmed to be other strains and not *Bacillus subtilis*. Weinstein and Colburn (1950) argued that while instance of such organisms have been reported; in a large number of cases they have been superimposed on a preexisting pathologic process, making evaluation of the role of the secondary invaders difficult.

Recurrent septicemia in an immunocompromised patient due to *Bacillus subtilis* was described by Oggionni *et al* (1998). A 73-year-old male patient with chronic lymphocytic leukemia was admitted to the hospital with hepatosplenomegaly and multiple pulmonary thickenings. Prior to hospitalization, the patient had been taking *Bacillus subtilis* spores which were discontinued upon admission. Blood cultures performed were positive for *Bacillus subtilis*, and although the episode resolved, the patient presented again with high fever and mental confusion two weeks later. The isolates from the blood cultures showed resistance to various antibiotics; however, the septicemia due to the probiotic strains of *Bacillus subtilis* could not be related directly to the patient's health condition. Therefore, no direct evidence of *Bacillus subtilis* causing the episode could be determined. Furthermore, literature search shows that various lactic acid bacteria, including *Bifidobacteria*, have been isolated as causes of bacteremia (Snydman, 2008). The list of organisms that have been associated with bacteremia includes *L. rhamnosus, L. plantarum, L. casei, Lactobacillus paracasei, Lactobacillus salivarius, L. acidophilus*, and many other *Lactobacilli*

(Snydman, 2008). In addition, *Lactococcus lactis* and *Leuconostoc* species, as well as *Pediococcus* species, have been demonstrated to cause bacteremia. *Bifidobacterium* species have also been isolated from the blood and in patients with endocarditis (Snydman, 2008). Though it is clear that bacteremia is not a result isolated to just *Bacillus* species, antibiotic resistance was assessed for *Bacillus subtilis* DE111 and none was found.

In the article by Spinosa *et al* (2000). the authors sought to confirm the identities of reported *Bacillus subtilis* strains causing infection in two published unrelated case reports. In 1996, a case report was published describing a cholangitis in polycystic kidney disease in a 15-year-old. Two years later, the case report by Oggioni (1998) was published describing the onset of recurrent septicemia due to *Bacillus subtilis*. In both cases, the relatedness of the bacterial strains to the disease was inconclusive. Due to the similarities in antibiotic resistance of the two strains, the authors sequenced the RNA of the clinical isolates and the probiotic strains. Results showed that there was 100% homology between the clinical isolates and the Italian probiotic, which was a mixture of antibiotic resistant mutants within the *Bacillus alcalophilus* group. Genome analysis was carried out to identify *Bacillus subtilis* DE111, showing its 99% homology to *Bacillus subtills* spizizenii and inaquosorum.

Stickel *et al* (2009) reported two patients suffering from hepatotoxicity after long-term consumption of an herbal supplement. Investigations led to the identification of *Bacillus subtilis* present in the supplement. The two patients suffered from cholestatic hepatitis and pruritus and cirrhosis, respectively. Though the bacteria were present and deemed contaminants in the supplement, further investigations of the bacterial isolates showed dose- and time-dependent hepatotoxicity on HepG2 cells increasing LDH leakage into the culture media. Although *Bacillus subtilis* was detected in some of the preparations; *Bacillus cereus,* a true pathogen, was detected in a batch of capsules from the same supplement company. Additionally, the authors mention that advanced fibrosis of the liver could relate to the long-term consumption and although hepatotoxicity was shown in the cytotoxicity studies performed with the *Bacillus subtilis* isolate, cytotoxicity of *Bacillus subtilis* DE111 was analyzed using four various cell types, including L-929, Caco-2, Vero, and Boar sperm cells. All studies showed that *Bacillus subtilis* DE111 has no cytotoxic effects, no hemolytic activity and is deemed safe for human consumption.

In an article by La Jeon *et al* (2012), an immunocompromised 71-year-old male patient with chronic obstructive pulmonary disease (COPD) and a history of mild drinking and pulmonary tuberculosis presented to the emergency department with a suspected esophageal perforation. Upon further investigation, bacteremia and mediastinitis were diagnosed after isolation of *Bacillus licheniformis* and *Bacillus subtilis* from pleural fluid and blood drained from a chest tube. The authors state that "In cases of *Bacillus* bacteremia, the majority of patients have a hematological malignancy, such as leukemia or lymphoma", and although the patient had no malignancies, no direct implication of this bacterial strain was shown within the article. Although the episode resolved after administration of antibiotics, the antibiotics were not specific to *Bacillus subtilis*, providing no definitive causation. This case was due to the bacterial strains present in the blood; the particular strain of *Bacillus subtilis* had α-hemolytic characteristics a known virulence factor among pathogenic organisms. Additionally, the *Bacillus licheniformis*

strain was resistant to multiple antibiotics, including penicillin, rifampicin, and erythromycin; the *Bacillus subtilis* DE111 strain does not have any hemolytic activity or antibiotic resistance. *Bacillus subtilis* DE111 was tested for hemolytic activity and antimicrobial susceptibility both through *in vitro* and molecular analysis. The strain was shown to have no toxicity against erythrocytes and possess no antimicrobial resistance genes.

Saleh *et al* (2014) described a *Bacillus subtilis* strain isolated from a burn wound in Iran. The authors state that *Bacillus subtilis* is one of the nonpathogenic bacteria which exists as a normal flora in humans and animals. However, the patient, a 26-year-old woman, with a third-degree burn presented with inflammation, swelling, pain and bleeding. Due to the infection, the patient suffered from septicemia. *Bacillus subtilis* was the organism suspected of causing the infection. After further investigation, the organism was identified through PCR and was shown to be resistant to cefotaxime and cefixime. However, the authors highlight that nonpathogenic bacteria may occasionally cause disease in humans. Therefore, septicemia is not just a possible outcome of *Bacillus subtilis* in the blood; cases involving other probiotics including *Lactobacillus rhamnosus* have also been reported in patients with clinical conditions (Doron and Stydman, 2015) as well as with *Bacillus coagulans* (Banerjee et al, 1988). Confirming the safety of *Bacillus subtilis* DE111, whole genome analysis of the strain was performed to determine toxin potential, in addition to biochemical testing such as hemolytic activity and cytotoxicity testing. No toxin-producing genes were found, no hemolytic activity was present and no deleterious effects were done on the cell lines tested.

The most recent case with *Bacillus subtilis*, involved a 51-year-old man who suffered from a grand mal seizure due to a lesion at the right parietal lobe surrounded by mild vasogenic edema. Tsonis *et al* (2018) reported that the poor oral hygiene with severe tooth decay and periodontal disease was the infectious foci that allowed an otherwise non-pathogenic bacteria, such as *Bacillus subtilis,* to gain access to the brain parenchyma. Because *Bacillus subtilis* are ubiquitous in nature and are normally found in soil and vegetation, as well as normal flora in humans, it is not an uncommon environmental organism. However, most bacterial brain abscesses, which are rare; are caused by aerobic bacteria such as *Streptococci* and anaerobic bacteria, such as *Bacteroides* and *Peptostreptococcus*. Furthermore, Patel and Clifford (2014) reported that contiguous infection may result from primary dental, sinus, ear infections, or mastoiditis; and represent 14% to 58% proportion of brain abscess. Although initially categorized as an immune-competent patient, the authors did propose the patient to be further evaluated for infectious foci and potential underlying immunosuppression.

It is clear that all reported cases of clinical infections with suspected *Bacillus subtilis* involvement occurred in subjects with one or more severe underlying diseases or health conditions. While these reports indicate that *Bacillus subtilis* has the potential to be an opportunistic pathogen in severely compromised subjects; it is equally clear that the genus is safe in healthy subjects and those with less severe medical conditions, where adverse events have never been reported. This conclusion is strongly supported by studies that have failed to discover any evidence of increased rates of clinical infection correlated with increased consumption of *Bacillus subtilis*. Positive blood cultures for *Bacillus subtilis* have also been regarded as indicators of serious or fatal

underlying disease in cases where etiologic strains were identified at the species level (a procedure that is not always done); the majority of cases were caused by antibiotic-resistant strains of *Bacillus subtilis*.

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11. Please provide information on the B. subtilis strains used for the toxicological studies and clinical trials described in the notice. Please comment briefly on how those studies support the safety of B. subtilis DE111 and comment on any differences between those strains and B. subtilis DE111 that would bear on safety.

Literature references to safety include other *Bacillus subtilis* strains as outlined below.

In the notice, the following strains have been referenced for animal toxicity: Richeux (2011) used *Bacillus subtilis* strain IAB/BS/03 (section 3.1 page 12). Nakamura *et al* (1999) used an unspecific strain of *Bacillus subtilis* (section 3.1 page 12). Kyoung-Hoon *et al* (2015) used *Bacillus subtilis* strain JNS (section 3.1 page 12) and Sorokulova *et al* (2008) analyzed *Bacillus subtilis* strain BS3, a strain with 99.8% identity to *Bacillus subtilis* 168 (section 3.1 page 13). All showed no toxicity in animals.

Further, literature searches for adverse effects of *Bacillus subtilis* consumption in humans were completed. The following strains were referenced: Naoko *et al* (2014) performed studies on a *Bacillus subtilis* strain *natto* (section 6.3 page 20), Novelli *et al* (1984) and Om and Yu (2017) used an unspecified strain of *Bacillus subtilis* (section 6.3 page 20), and Lefevre *et al* (2015) analyzed *Bacillus subtilis* strain CU1 (section 6.3 page 20). Lastly, Horosheva *et al* (2014) tested *Bacillus subtilis* BS3 (section 6.3 page 20). All showed no adverse effects in humans after consumption.

Literature searches for acute toxicity were also performed. Strains including *Bacillus subtilis* BS3 by Sorokulova *et al* (2008), *Bacillus subtilis* species *natto* by Hong *et al* (2008) and *Bacillus subtilis* RO179 by Tompkins *et al* (2008) were analyzed and no acute toxicity effects were observed (section 7.1 pages 23 & 24).

Extensive testing was performed to confirm that *Bacillus subtilis* DE111 was safe and did not contain any kind of toxin genes associated with other *Bacillus* species. The tests performed specifically on DE111 include: (1) full genome analysis to determine if any deleterious genes were present (pages 45-54), (2) PCR to confirm enterotoxins and emetic toxins were not present (pages 55-57), (3) complete genome sequence of proteins against known allergen database with no allergen potential found (pages 83-84), (4) gene sequence alignment to a database of antibiotics with no antibiotic resistance genes found (pages 58-59); (5) cytotoxicity on Vero monkey kidney cells (pages 76-79), (6) CaCo2 human cells (pages 134-135), and (7) L-929 mice cells (pages 74-75) subjected by *Bacillus subtilis* DE111 with no cytotoxic effects observed; (9) hemolytic activity (page 80-81), (10) boar sperm motility (page 82), (11) zones of inhibition, and (12) minimum inhibitory concentrations for antimicrobial susceptibility of *Bacillus subtilis* DE111 (pages 60-71). Furthermore, four human clinical trials were conducted using *Bacillus subtilis* DE111 (pages 85-133) with no adverse events reported; complete metabolic panels showed all participants remained within clinically normal parameter ranges, supporting the conclusion that DE111 is safe for humans.

Sincerely,

(b) (6)

Catherine Adams Hutt, PhD, RD, CFS
President, RdR Solutions Consulting, LLC

From:	Catherine Adams
To:	Morissette, Rachel
Cc:	John Deaton; Anamaria Cuentas
Subject:	Re: follow-up questions for GRN 000831
Date:	Friday, May 24, 2019 10:36:03 AM
Attachments:	image002.png FDA GRAS Response 052419.docx

Rachel,

Attached are our responses to each of your questions. Please let me know if you have additional questions or wish me to walk you through the replies. It would be my pleasure.

Catherine Adams Hutt, PhD, RD, CFS
RdR Solutions
4568 Elm Bottom Circle
Aubrey, TX 76227
and 124 S. Fairfax
Alexandria, VA 22314
630-605-3022

On Tue, May 21, 2019 at 9:37 AM Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:

> Dear Catherine,
>
> Our microbiologist had some follow-up questions regarding your April 30 amendment. Please provide responses within 5 business days.
>
> 1. The specifications provided in the notice and those provided in the amendment in response to question #7 do not match the specifications given in the certificates of analyses for the individual lots in response to question #8. Please clarify this discrepancy and which specifications are being used.
>
> 2. The table in response #7 shows the specification for *Salmonella* per 25 g sample, but the response to question #9 says 10 g sample. Please confirm which set of specifications you are using.
>
> 3. Regarding your response to question #10, please provide a statement that while opportunistic infections have been reported, *B. subtilis* DE111 is safe for consumption.
>
> 4. Please confirm by providing a statement that the results of the testing of *B. subtilis* DE111

discussed in response #11 demonstrate that the DE111 strain does not differ from the strains used in the toxicological testing in any ways that impact safety; therefore, these studies support the safe use of *B. subtilis* DE111.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

Center for Food Safety and Applied Nutrition
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rachel.morissette@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

Catherine Adams Hutt, PhD, RD, CFS
4568 Elm Bottom Circle
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630-605-3022

May 24, 2019

Rachel Morissette, Ph.D.
Consumer Safety Officer
FDA Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review

Dear Dr. Morissette,

We submit the following information in response to your email of May 21, 2019 with four additional requests for information regarding Deerland Probiotics and Enzymes (Deerland)'s GRAS Notice GRN 000831.

1. The specifications provided in the notice and those provided in the amendment in response to question #7 do not match the specifications given in the certificates of analyses for the individual lots in response to question #8. Please clarify this discrepancy and which specifications are being used.

The specifications provided in response to question #7 are newly adopted specifications unique for infants that were implemented after receipt of the comments requesting addition of *Enterobacter sakazakii.*

When testing for limits of microbiological contaminates, the laboratory will often plate to the lowest common specification; which is ≤100 CFU/g for yeast and mold, ≤30 CFU/g for *Enterobacter*, and ≤30 CFU/g for coliforms. The laboratory COA reported the values at the dilutions tested as both the specification and result – as no growth was observed. If DE111 did not meet the more restrictive requirements from the initial test, additional dilutions would be conducted to determine compliance to the Deerland specification.

DE111 is only offered in a powdered form. Analysis of pH and specific gravity testing are reserved for liquid products and are, therefore, marked N/A.

2. The table in response #7 shows the specification for *Salmonella* per 25 g sample, but the response to question #9 says 10 g sample. Please confirm which set of specifications you are using.

There are two sets of specifications, one for adults and children, and one for infants. The table in response #7 shows the infant formula specification which tests for *Salmonella* per 25 g sample. The response for question #9 and the specification provided within the Notification are for adult/child specifications, which tests for *Salmonella*, *E. coli* and *Staphylococcus* per 10 g.

3. Regarding your response to question #10, please provide a statement that while opportunistic infections have been reported, *B. subtilis* DE111 is safe for consumption.

We have researched and considered all reported cases of bacteremia involving *Bacillus subtilis* or that were attributed to *Bacillus subtilis*. As we explained in our response to question #10, we declare that while opportunistic infections have been reported, *Bacillus subtilis* DE111 is safe for consumption.

4. Please confirm by providing a statement that the results of the testing of *B. subtilis* DE111 discussed in response #11 demonstrate that the DE111 strain does not differ from the strains used in the toxicological testing in any ways that impact safety; therefore, these studies support the safe use of *B. subtilis* DE111.

Toxicity testing and results included in the GRAS Notification GRN 000831 and summarized in response to question #11 were performed using *Bacillus subtilis* strain DE111, supporting its safe use in humans. We declare that there were no strain differences between the substance evaluated for toxicity and *Bacillus subtilis* DE111 that would impact safety.

Sincerely,

(b) (6)

Catherine Adams Hutt, PhD, RD, CFS
President, RdR Solutions Consulting, LLC

From:	Morissette, Rachel
To:	Catherine Adams Hutt
Subject:	RE: issue with review of GRN 000831
Date:	Saturday, July 20, 2019 1:57:00 PM
Attachments:	image007.png

This email is sufficient. We'll proceed with the review of your notice with the remaining intended uses.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

From: Catherine Adams Hutt <cadams@rdrsol.com>
Sent: Saturday, July 20, 2019 1:46 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Subject: Re: issue with review of GRN 000831

We intend to withdraw the intended use in alcoholic beverages. What do I need to do to formalize this withdrawal from intended uses and allow FDA to move ahead with evaluation absent reference to alcoholic beverages.

Thank you.
Catherine

Sent from my iPhone

On Jul 20, 2019, at 12:35 PM, Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:

> This is pulled directly out of your notice on page 6.
>
> For adults and children, *Bacillus subtilis* DE111 may be added to: baked goods and baking mixes; alcoholic beverages; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fats and oils; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy and cough drops; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products;

processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes*; sugar; and sweet sauces, toppings, and syrups.

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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From: Catherine Adams Hutt <cadams@rdrsol.com>
Sent: Saturday, July 20, 2019 1:34 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Subject: Re: issue with review of GRN 000831

We have no intended use in alcoholic beverages. I'm not sure where this is coming from? Im happy to clarify, but no intended use in alcoholic beverages.

Thank you.

Sent from my iPhone

On Jul 20, 2019, at 10:54 AM, Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:

> Dear Catherine,
>
> I need to bring to your attention an issue regarding the intended use of your *B. subtilis* DE111 ingredient in alcoholic beverages. Alcoholic beverages are not considered "healthy" (hence the warning label) and cannot be associated with any type of health-related claim, as is implied by reference to its use as a "probiotic" in GRN 000831. Including this intended use in your GRAS notice will require our office to communicate this intended use to the Alcohol and Tobacco Tax and Trade Bureau to address the policy implications and labeling. Therefore, this issue would not be resolved within the statutory 180-day deadline for a GRAS notice and we would not be able to move forward with a no questions letter. Therefore, our recommendation would be to withdraw the intended use in alcoholic beverages, in which case we can move forward with the remaining intended uses. Please let me know by close of business July 26, 2019 if you end to withdraw the intended uses in alcoholic beverages, withdraw the GRAS notice in its entirety, or proceed with the notice as is, in which case we would issue a no basis letter.
>
> Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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rachel.morissette@fda.hhs.gov

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From: Catherine Adams
To: Morissette, Rachel
Subject: Re: question about specifications
Date: Tuesday, July 23, 2019 10:19:06 AM
Attachments: image001.png

We are providing additional language to clarify Deerland's intent, in response to your question.

"Deerland states that *B. subtilis* DE111 spore preparation will be manufactured to meet these specifications, including the specification for *Cronobacter* for infant formula."

Please advise if you need any more information or clarification. Thank you for your timely response.

Best regards,
Catherine

Catherine Adams Hutt, PhD, RD, CFS
RdR Solutions
4568 Elm Bottom Circle
Aubrey, TX 76227
and 124 S. Fairfax
Alexandria, VA 22314
630-605-3022

On Mon, Jul 22, 2019 at 2:59 PM Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:

> No, we do not have an issue with the specification itself. However, batch analyses for the *Cronobacter* spec were not provided in your amendments; therefore, we are unable to comment in the letter on whether your manufacturing method can meet the specifications that you provided for *Cronobacter*. The proposed language would clarify that point without having to specifically address the batch analyses in the response letter.
>
> Best,
>
> Rachel
>
> ---
>
> **Rachel Morissette, Ph.D.**
> *Regulatory Review Scientist*
>
> **Center for Food Safety and Applied Nutrition**
> **Office of Food Additive Safety**

U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

From: Catherine Adams Hutt <cadams@rdrsol.com>
Sent: Monday, July 22, 2019 3:54 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Subject: Re: question about specifications

Before I confirm, which I am happy to do: can you please tell me if there is any part of the specification that you find objection with?

Thank you.

Sent from my iPhone

On Jul 22, 2019, at 1:21 PM, Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:

> Dear Catherine,
>
> Regarding the specifications that Deerland provided in its original notice and subsequent amendments, can you please confirm that the following statement is correct:
>
> "Deerland states that *B. subtilis* DE111 spore preparation will be manufactured to meet these specifications."
>
> Thank you for your attention to this matter.
>
> Best regards,
>
> Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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rachel.morissette@fda.hhs.gov

    

From: Catherine Adams
To: Morissette, Rachel
Subject: Re: request for clarification on GRN 000831
Date: Wednesday, August 07, 2019 3:27:59 PM
Attachments: image013.png

As Deerland's agent in this matter and on behalf of Deerland Probiotics and Enzymes, we concur with the changes as presented in your e-mail of today.

Thank you for your call and your time.

Best regards,
Catherine

Catherine Adams Hutt, PhD, RD, CFS
RdR Solutions
4568 Elm Bottom Circle
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and 124 S. Fairfax
Alexandria, VA 22314
630-605-3022

On Wed, Aug 7, 2019 at 3:21 PM Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:

> Dear Catherine,
>
> Thank you for speaking with me today regarding the terminology used to described your intended use in GRN 000831. As I explained over the phone, we are asking Deerland Probiotics if they concur with the following description of the intended use as an ingredient and not a “probiotic” (which has no regulatory definition) or “delivery system” and that the intended use of *Bacillus subtilis* DE111 is GRAS, and not the ingredient itself.
>
> Therefore, does Deerland Probiotics concur that:
>
> The subject of the notice is *Bacillus subtilis* DE111 for use as an ingredient at a level not greater than 2 x 10^8 colony forming units (CFU)/100 mL in cow’s-milk and soy-based non-exempt infant formula for term infants and at levels from 1 x 10^6-1 x 10^{10} CFU/serving in conventional foods, including baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fats and oils; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; soft/hard candy and cough drops; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk and milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soups and soup mixes;2 sugar; and sweet sauces, toppings, and syrups. The

notice informs us of Deerland's view that these uses of *B. subtilis* DE111 are GRAS through scientific procedures.

Thank you for your attention to this matter.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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