TOLERANCE AND EFFICACY OF THE PROBIOTIC DE111™ DELIVERED IN CAPSULE FORM

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Background
The term probiotics is derived from the Greek meaning "for life", and are defined as organisms, when ingested in adequate amounts, exert a health benefit to the host. Probiotic supplements have shown benefit in increasing frequency and efficiency of bowel movements, immunity, digestion and as competitive exclusion agents.

Objectives
The objectives of this clinical study were to determine if daily consumption of *Bacillus subtilis* Strain DE111 at a 5 x 10^9 CFU/dose per day is safe for human consumption and efficacious as a probiotic.

Design
The tolerance and efficacy of encapsulated *Bacillus subtilis* Strain DE111 at a 5 x 10^9 CFU/dose per day was assessed in an average 20-day double-blind, randomized, placebo based study.

Results
The majority of the blood parameters remained within normal ranges throughout; however, fasted serum glucose levels in the probiotic group ($\alpha \leq 0.05$; P = 0.012) were significantly reduced. There were no significant differences presented in the average number of bowel movements per day within the probiotic group. There was a significant increase in the average number of bowel movements per day within the control group ($\alpha \leq 0.05$; P=0.015). Significant differences in microbe colonization were present for *B.subtilis* and *Bifidobacterium* in the fecal colony counts.

Conclusion
Daily consumption of *Bacillus subtilis* Strain DE111 at a 5 x 10^9 CFU/dose per day can be recognized as a safe efficacious probiotic.

INTRODUCTION

The human gastrointestinal microflora is a complex ecosystem of approximately 300-500 bacterial species compromising nearly two million genes (Bengmark 1998 and Neish 2009). This is commonly referred to as the microbiome. The vast amount of bacteria in the gut is in the vicinity of 10 times greater than the cells in the human body. At birth, the intestinal tract is sterile, but upon the consumption of food, bacteria begins to populate the gastrointestinal tract. The microflora that reside within the human gut generally fall into one of three different symbiotic categories: mutualistic (microbe benefits and host benefits: +/+), communalistic (microbe benefits with no effect on the host: +/o or neither the microbe nor the host are affected: o/o), and pathogenic (the microbe benefits and the host is harmed: +/-) (Hooper 2001 and Neish). The interactions between the host's immune system and the nonpathogenic constituents of the microbiota plays an important role in protecting the host from colonization by pathogenic species through immunity and competitive exclusion agents.

Because the composition of the microbiota is influenced by a variety of factors including diet, socio-economic conditions, age, and most importantly, the use of antibiotics, the ratio of good bacteria to bad bacteria is a critical measure in determining overall health. Gut commensals, such as probiotics, exhibit various beneficial effects for the host (Rolfe 2000). Probiotics are live microorganisms passing through or residing in the human gut with low or no pathogenicity and exhibit beneficial effects for the host (Bengmark 1998, Geier et al. 2007, Rauch and Lynch 2012, Rolfe 2000). Probiotic supplementation has shown positive results for relief of various ailments such as: antibiotic-associated diarrhea, constipation, allergies, and diabetes (Al-Salami et al. 2008, Fooks et al. 1999, Goldin and Gorbach 2008, Ranadheera et al. 2009, Rauch and Lynch 2012, and Rolfe 2000). Probiotics have also exhibited protective properties by producing inhibitory substances, competitive inhibition of pathogenic bacteria, degrading toxin receptors, and stimulating the immune system (Casula and Cutting 2002, Fooks et al. 1999, Geier et al. 2007 and Rolfe 2000).

Common probiotics are lactic acid producers such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* due to their resistance to gastric acids, bile salts, and pancreatic enzymes (Rauch and Lynch 2010,

and Rolfe 2000). 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* (Rolfe 2000 and Bengmark 1998).

However, not all probiotics are lactic acid bacteria. *Bacillus subtilis* spores have been used as probiotics, competitive exclusion agents, and prophylactics for human and animal consumption (Casula & Cutting 2002). *Bacillus* strains are increasingly popular around the world (Mercenier et al., 2003; Sanders et al., 2003) and have been long used in Eastern Europe for prophylactic and therapeutic use against several gastrointestinal disorders (Sorokulova et al., 2008). *Bacillus* species play a significant role in the gut because of their high metabolic activity. They support healthy gut function and stimulate normal microflora for the gut. *Bacilli* also produce amino acids (Simmov, 1992) and vitamins (Walter & Bacher, 1977; Bentley & Meganathan, 1982). Some strains effectively degrade cholesterol *in vitro* (Kim *et al*., 2002) and reduce low-density lipoproteins, hepatic total cholesterol, and triglycerides after oral adminstration in animals (Paik *et al*., 2005).

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

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

Over a period of many centuries these bacteria have been used for preparation of alkaline-fermented foods (Wang J& Fung DYC, 1996). *Bacillus* species are the major microflora in soybeans and are responsible for their fermentation into soy food products and condiments (Ray *et al*., 2000; Inatsu *et al*., 2006). In Japan, a culture of *Bacillus subtilis* subsp. *natto* is used to produce Natt*ō*, a popular food made by fermenting cooked soybeans (Katz & Demain, 1977). *Natt*ō is a traditional Japanese food made from soybeans fermented with *Bacillus subtilis*. In addition, previous studies have shown that *Bacillus subtilis* subsp. *natto* increased general performance and immune function of preweaning calves (Sun *et al*., 2010) and has some fibrinolytic and antithrombotic activity (Omura et al., 2005), Studies in chickens showed that *Bacillus subtilis* inhibited pathogenic microorganism growth (Fritts et al., 2000; Teo and Tan, 2005), increased digestive enzyme activity, and reduced the yield of ammonia (Samanya and Yamauchi, 2002), which in turn promoted fowl growth performance (Fritts et al., 2000; Teo and Tan, 2005). The probiotic used in this clinical study, *Bacillus subtilis* str. *DE111* was sequenced and found to have an Average Nucleotide Identity (ANI) score of 92.9% in common with *Bacillus subtilis* subsp. *natto* str. BEST195, indicating the high degree of similarity between the two strains and their shared functionalities.

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 *B. subtilis*. Tolerance is assessed through analysis of blood biomarkers within comprehensive clinical metabolic and liver panels, and immunoreactive C-reactive protein (CRP), a substance that reflects acute stress (Johnstone 2014). 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.

METHODS AND MATERIALS

Study Design

Forty-one subjects were recruited for participation through print and local social media advertisements and signed the informed consent approved by the Institutional Review Board (IRB) for the Protection of Human Subjects, at the University of Wisconsin-La Crosse (Appendix A). This probiotic supplement study was performed in a randomized double-blind, placebo-based design with daily probiotic or placebo capsule intake by subjects for an average of 20 days (range of 15-23 days). Subjects were randomly assigned to probiotic supplement or placebo control groups (Table 1). Subject ages ranged from 19-42 years of age. One subject withdrew from the study after two days of capsule consumption.

Subject Dynamics- Criteria for inclusion in the study were adult age (≤ 18 years of age at time of participation), no reported illnesses at the time of recruitment, and no reported us of antibiotics for at least seven days prior to recruitment. Subjects would be excluded if antibiotic use were reported at any point throughout the study.

Questionnaire Design- The questionnaire used in this clinical was approved by the IRB at the University of Wisconsin-La Crosse. This questionnaire was designed to provide a brief health history and gauge gastrointestinal symptoms (Appendix B).

Prior to Capsule Consumption

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

Blood Sample- Trained phlebotomists used routine veniouncture procedures with serum separation tubes to collect blood samples from arm veins. Each subject provided a 12-hour fasted blood sample of 15 mL. Blood was allowed to clot for 20 minutes at room temperature. The collection tubes were spun at 2,500 rpm for 15 minutes, which allowed for serum separation. The serum was poured off into two analysis tubes and sent to Gundersen Health System, La Crosse, WI, for clinical laboratory analysis of comprehensive metabolic, and lipid panels and C-reactive protein (CRP) (Table 2). Samples were analyzed using a Cobas 6000 (Roche/Hitachi, Indianapolis, IN) automated clinical chemistry immunoassay system.

Bowel Movement Sample- Subjects were asked to refrain from consuming diuretics (including caffeine) and laxatives for this sample. All subjects provided his or her first natural bowel movement of the day in a Fisherbrand™ Commode Specimen Collection System (Thermo Fisher, Catalog number: 02-544-208, Waltham, MA). Samples were transported from the subject's home to the Health Science Center at the University of Wisconsin-La Crosse campus in supplied bags, and were processed immediately upon arrival. At least 200 mg subsamples were placed in sterile 2 mL collection tubes and stored at -80°C until DNA extraction or plating was executed.

Capsule Consumption

Subjects were instructed to take the assigned capsule once per day, with or without food. If a dose was missed, subjects were instructed to take two capsules the following day. Recurring incidences of missed doses were to be reported to the project leader; none were reported. Subjects were instructed to complete a daily food-intake record, which was to include any and all alcohol consumption throughout the course of the study. The probiotic capsules, provided by Deerland Enzymes Inc., Kennesaw, GA, contained approximately 5×10^9 colony forming units (CFU)/capsule of *Bacillus subtilis* Strain *DE111* and the placebo capsules contained maltodextrin.

Final Day of Capsule Consumption

All subjects completed the provided gastrointestinal questionnaire to gauge final gastrointestinal symtoms (Appendix B). At this time, subjects handed in their completed booklets and were given $100 compensation for participation and completion of the study.

Blood Sample- Blood was sampled and analyzed (Table 2) as previously described in the Prior to Capsule consumption section.

Bowel Movement Sample- Fecal samples were collected and analyzed as previously described in the Prior to Capsule consumption section.

Statistical Analyses
Samples were analyzed using IBM SPSS Statistics for the Wilcoxon Signed-rank test and T-test for independent means. Analysis was performed within subjects factor of time (pre-versus post-capsule consumption) and between subjects factor of capsule type (probiotic versus placebo control group).

Fecal Plating
Fecal plating was 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 *B. subtilis, E. coli, L. acidophilus, B. longum,* and *C. albicans.*

Even though nutrient specific agar plates were used to grow specific strains, other strains are capable of growing and contaminating these plates. *B. cereus* agar base plates were used to grow *B. subtilis*. Both strains showed growth during the fecal plate process. MacConkey agar was used for *E. coli* growth and is selective for gram-negative bacteria and lactose fermenters (i.e. *Escherichia, klebsiella, Enterobacteri,* etc.). Rogosa SL agar was used *Lactobacilli* growth. Liver veal agar is selective for anaerobic bacteria and fastidious aerobic pathogens and was used for *Bifidobacterium* growth. DRBC agar is selective and was used for the detection of yeast such as *Candida.* For selective media agar plate information and culture conditions, see Appendix D.

RESULTS
Blood Analysis
The comprehensive metabolic and lipid panels revealed several differences between the probiotic group and the control group. There was a significant time by capsule interaction in serum fasting glucose levels present in the probiotic group ($\alpha \leq 0.05$; $P = 0.012$) (Figure 1). Paired T-test indicated a significant decrease in serum glucose in the probiotic group ($\alpha \leq 0.05$; $P = 0.001$), 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 ($\alpha \leq 0.05$; $P \leq 0.042$) (Figure 2). Bilirubin significantly decreased from pre to post in the probiotic group ($\alpha \leq 0.05$; $P \leq 0.046$), but was not significant in the control group (Figure 3). 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 ($\alpha \leq 0.05$; $P \leq 0.025$) (Figure 2). There was no significant variation from the normal range of CRP by time or capsule (Figure 5).

Gastrointestinal Symptom Questionnaire
While there were no significant differences in gastrointestinal questionnaire answers taken before and after (pre and post) capsule consumption between the probiotic and control 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 control group reported a slight increase in symptoms in these questions (Figure 6). 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 (Figure 7).

Bowel Movement Records
The control group had a significant increase in average bowel movements per day when compared to the probiotic group over the course of capsule consumption ($\alpha \leq 0.05$; $P = 0.015$) (Figure 8). There was no significant difference in average daily stool type, as rated using the Bristol Stool chart, between groups throughout the course of capsule consumption (Figure 9).

Fecal Plate Counts
Fecal plate counts are displayed in Figures 10-12. 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 subjects factor of capsule type (Probiotic versus placebo control group) ($\alpha \leq 0.05$; $P = 0.049$). Subjects who were administered the placebo demonstrated a decrease in intestinal levels of the probiotic *Bifidobacterium*, while those wh were administered the probiotic experienced a significant increase with respect to time within the probiotic group ($\alpha \leq 0.10$; $P = 0.10$) and a significant difference between factors of capsule type (Probiotic versus placebo control group) ($\alpha \leq 0.10$; $P = 0.08$). Subjects who were administered the placebo demonstrated a numerical increase in levels of *E.coli* while those who were administered the Probiotic experienced a slight decrease in *E.coli*. No noticeable differences were displayed for either *Lactobacillus* or yeast.

DISCUSSION

Limitations of the Study

The study population was predominantly a sample of forty 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 exam week. During the time of final exams and before the final sample collections, there was an increase in consumption of alcohol, candy, and fatty foods.

Blood Parameters

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 *B. subtilis* that, when fed to bovine calves, improved diabetic conditions by improving insulin sensitivity (Lee et al. 2013). In addition, freeze-dried cultures of *L. acidophilus*, *B. lactic*, and *L. 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.

Bowel Movement Records

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 (Hong et al. 2005, Jain and Chaudhary 2014, Saarela et al. 2000, and Schrezenmeir and de Vrese 2001, Saarela et al. 2000). 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 (Figure 9).

CONCLUSION

Daily ingestion of one capsule containing approximately 5 x 10^9 colony forming units (CFU)/ capsule of *B. subtilis* 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 did increase less in the probiotic group. Triglycerides levels were maintained in the probiotic group, but increased significantly in the control group. Finally, consumption of *B. subtilis* in the manner described herein, may improve glucose tolerance, corroborating the findings of non-human animal *in vivo* and *in vitro* studies by Al-Salami et al. (2008) and Lee et al. (2013), respectively. This probiotic is a safe, efficacious dietary supplement for immunity, digestive health, and as a competitive exclusion agent. Daily consumption of the *B. subtilis* probiotic supplement resulted in a significant effect on gut microflora measured prior to and after capsule consumption in regards to *B. subtilis* and *Bifidobacterium*.

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LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

TABLES

Table 1: Subject Demographics for Probiotic and Control Groups

Gender	Probiotic Group	Control Group	Mean Age (years)
Male	11	7	23.6 3 5.3
Female	10	13	22.5 3 2.4
Total	21	20	23.0 3 3.9

*Subject ages ranged from 19-42 years of age. One subject withdrew from the study after two days of capsule consumption.

Table 2: Components of Metabolic and Lipid Panels

	Albumin	ALP	ALT	AST	BUN	Calcium	Chloride	CO2	Creatinine	Glucose
Comprehensive Metabolic Panel	●	●	●	●	●	●	●	●	●	●
Lipid Panel										
C Reactive Protein (CRP)										

	Potassium	Sodium	Bilirubin	Protein	Cholesterol	HDL	LDL	NonHDL	Triglyceride	CRP
Comprehensive Metabolic Panel	●	●	●	●	●	●			●	
Lipid Panel					●	●	●	●	●	
C Reactive Protein (CRP)										●

FIGURES

Figure 1: BUN, Creatinine, Protein, Albumin, and Glucose: Values are expressed as mean ± 3 standard error of the mean.
††: significant time by capsule interaction, significant difference pre to post in probiotic group only by paired T-test.
*BUN = Blood Urea Nitrogen

Group	BUN Pre (8-26 mg/dL)	BUN Post (8-26 mg/dL)	Creatinine Pre (0.6-1.1 mg/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 ± 0.2	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 ± 0.91	6.87 ± 0.11	4.46 ± 0.06	4.51 ± 0.08	86.2 ± 1.3	85.9 ± 1.0

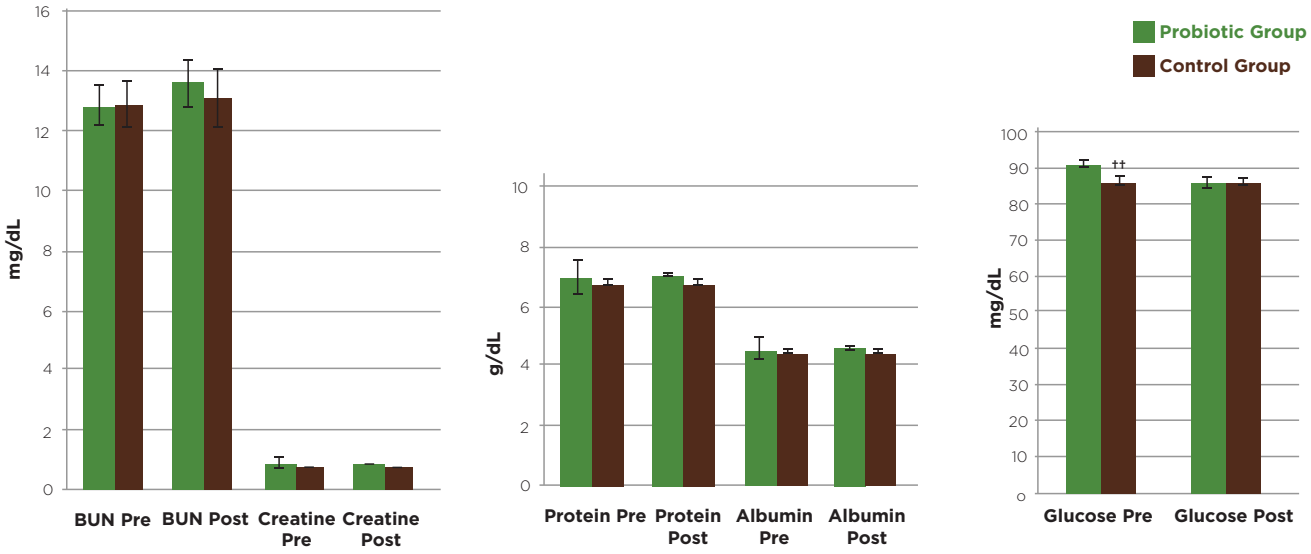


Figure 2: Lipid Panel: Values are expressed as mean ± 3 standard error of the mean.
†: no significant time by capsule interaction, but significant difference in pre to post in the placebo group only with paired T-test analysis.

Group	†Cholesterol Pre (<200 mg/dL)	†Cholesterol Post (<200 mg/dL)	†Triglyceride Pre (33-137 mg/dL)	†Triglyceride Post (33-137 mg/dL)	HDL Pre (40-60 mg/dL)	HDL Post (40-60 mg/dL)	†LDL Pre (<130 mg/dL)	†LDL Post (<130 mg/dL)	Non HDL Pre (LDL + 30 mg/dL)	Non HDL Post (LDL + 30 mg/dL)
Probiotic	165.0 3 7.0	169.4 3 6.7	98.4 3 15.3	97.9 3 5.7	59.5 3 3.3	60.1 3 3.2	85.9 3 5.6	89.6 3 5.4	105.5 3 6.6	104.3 3 5.9
Control	160.0 3 7.0	169.0 3 6.7	87.5 3 11.9	103.8 3 7.6	59.5 3 3.1	59.4 3 2.9	83.1 3 6.5	88.9 3 5.9	100.6 3 7.0	109.6 3 6.5

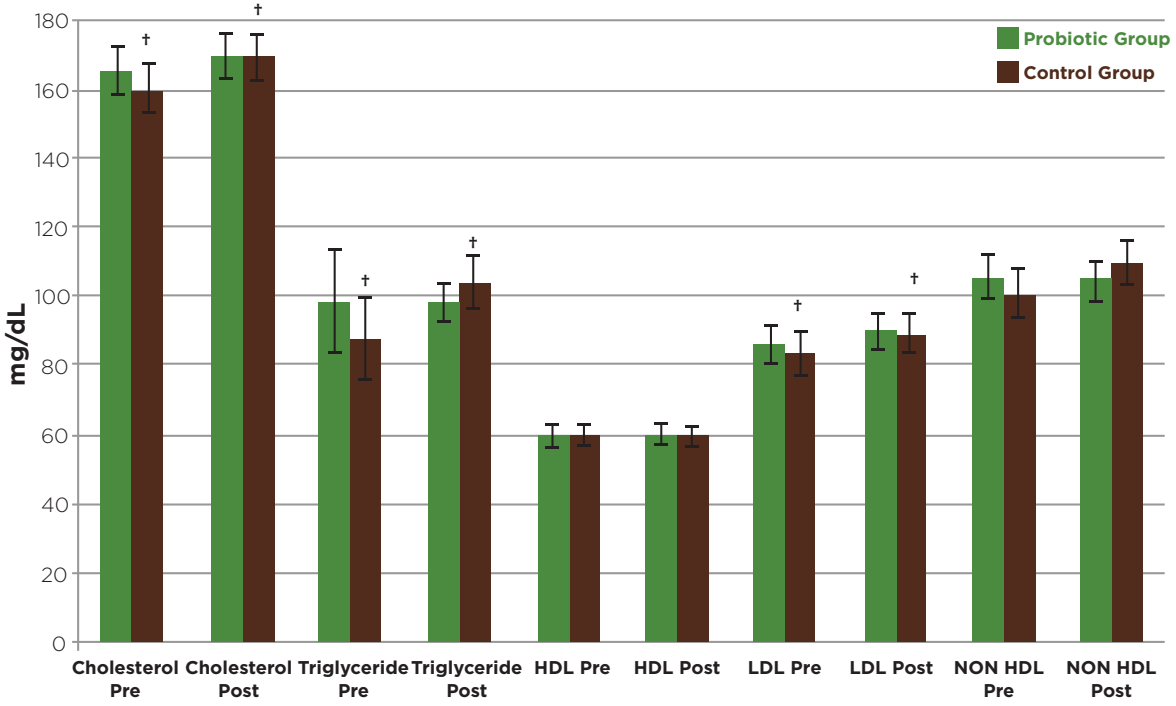


Figure 3: Bilirubin, ALKP, AST, and ALT: Values are expressed as mean ±3 standard error of the mean.
†: significant difference with respect to time.
*ALP = Alkaline Phosphatase, ALT= Alanine Transaminase, and AST = Aspartate Transaminase

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

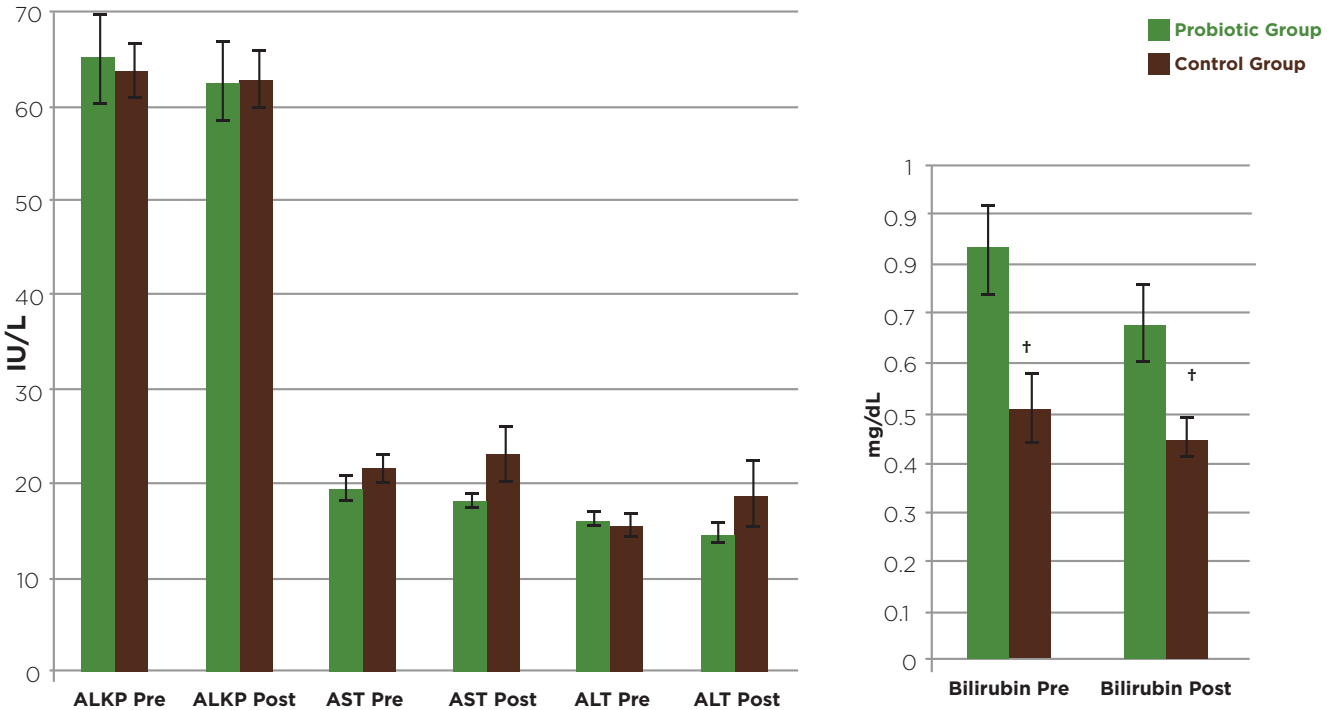


Figure 4: Electrolyte Panel: Values are expressed as mean ±3 standard error of the mean.
†: significant difference with respect to time

Group	Na^+ Pre (135-146 mmol/L)	Na^+ Post (135-146 mmol/L)	K^+ Pre (3.4-5.0 mmol/L)	K^+ Post (3.4-5.0 mmol/L)	CL^- Pre (96-108 mmol/L)	CL^- Post (96-108 mmol/L)	CO_2 Pre (22-29 mmol/L)	CO_2 Post (22-29 mmol/L)	†Ca^{2+} Pre (85-104 mg/dL)	†Ca^{2+} Pre (85-104 mg/dL)
Probiotic	138.6 ± 0.3	140.0 ± 0.4	4.11 ± 0.04	4.11 ± 0.05	101.6 ± 0.3	101.4 ± 0.3	25.1 ±0.3	25.8 ± 0.3	9.27 ± 0.05	19.41 ± 0.06
Control	139.6 ± 0.4	140.5 ± 0.3	4.16 ± 0.07	4.13 ± 0.05	101.9 ± 0.3	102.1 ± 0.3	25.9 ± 0.3	26.7 ± 0.3	9.37 ± 0.06	9.49 ± 0.07

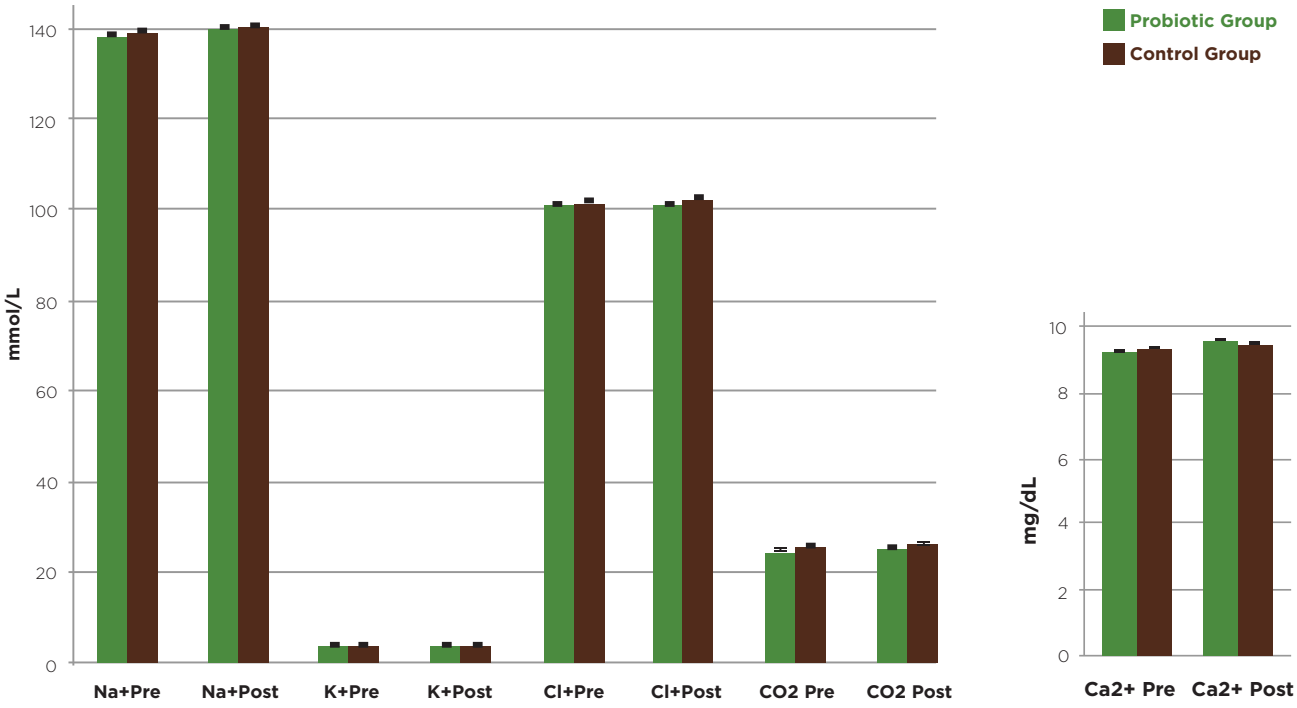


Figure 5: C-Reactive Protein (CRP): Non-stressed range for CRP was defined as ≤ 0.8 mg/dL by the Gundersen Health System clinical lab.

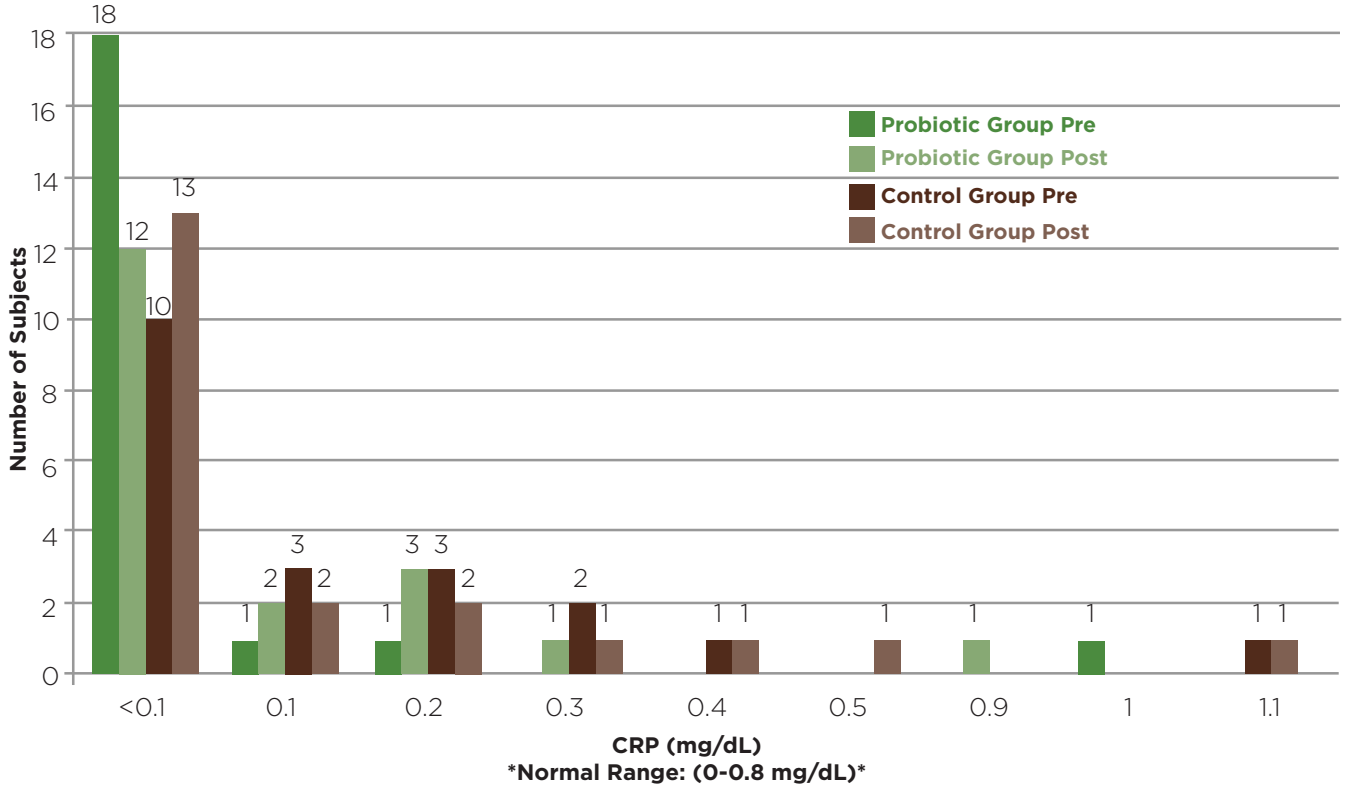


Figure 6: Answers to questions 1-8 on the gastrointestinal symptom questionnaire. For questions corresponding to the questionnaire, please refer to Appendix B.

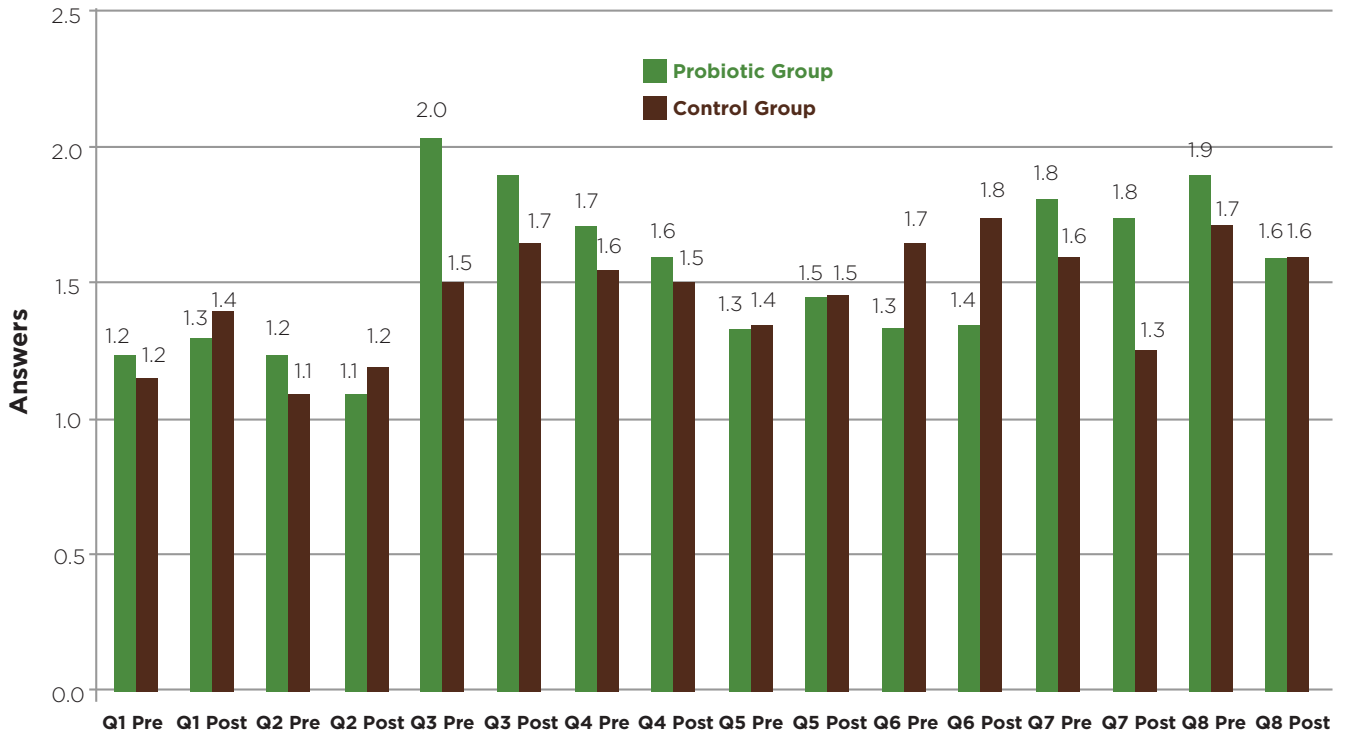


Figure 7: Answers to questions 9-15 on the gastrointestinal symptom questionnaire. For questions corresponding to the questionnaire, please refer to Appendix B.

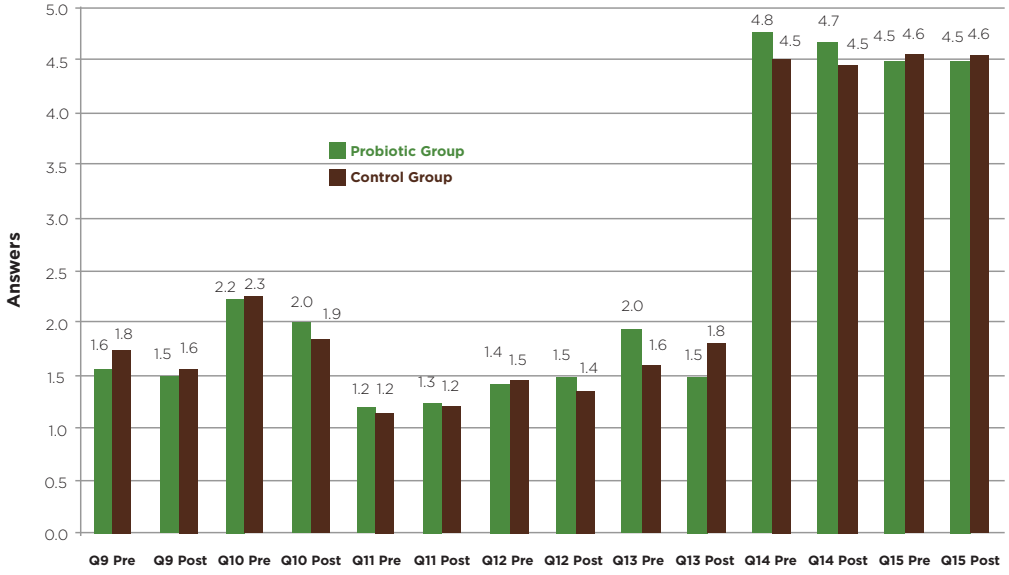


Figure 8: Average number of bowel movements per day between the probiotic and control groups. Subjects in the probiotic group had significantly more daily bowel movements ($\alpha \leq 0.05$; P = 0.015).

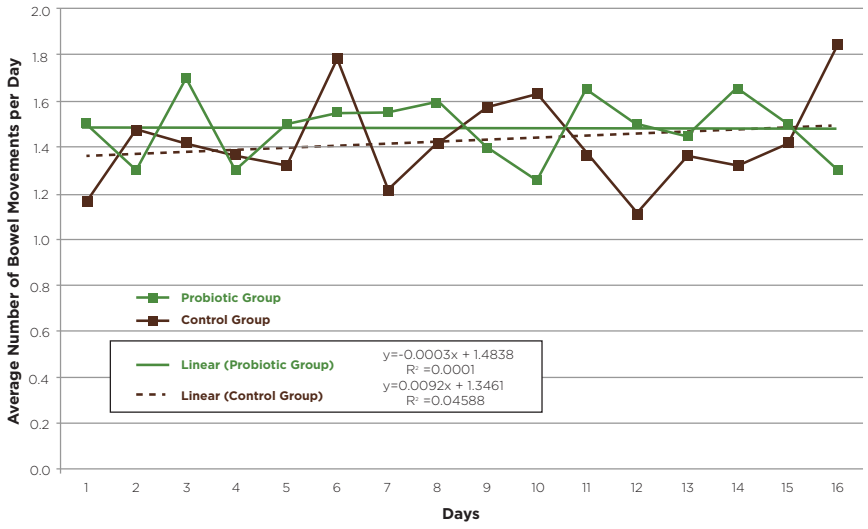


Figure 9: Average stool type per day between the probiotic and control groups. Stool types were based on the Bristol stool chart and did not change significantly in either group over time.

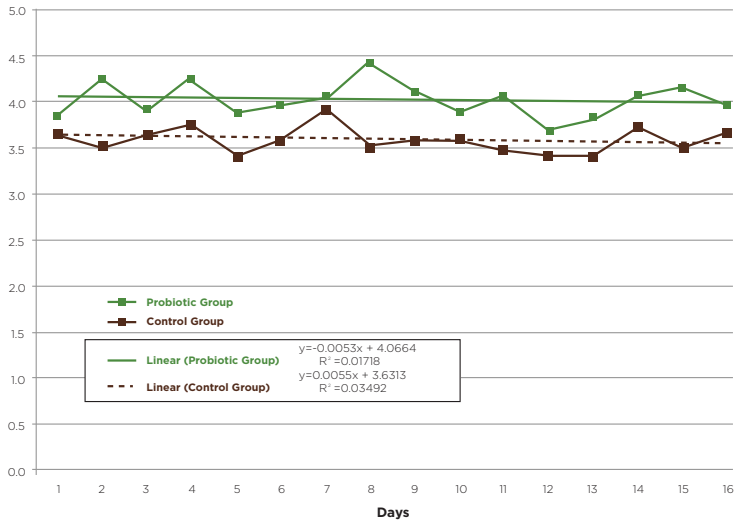


Figure 10: *Bacillus subtilis* Fecal Counts. There was a significant difference with respect to time within the probiotic group ($\alpha \leq 0.05$; P=0.0053) and a significant difference between subjects factor of capsule type (Probiotic versus placebo control group) ($\alpha \leq 0.05$; P=0.049)

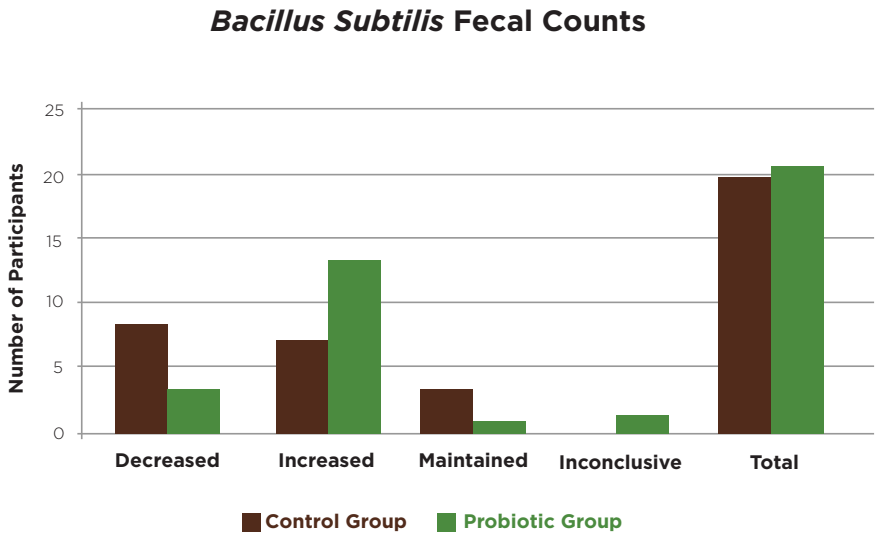


Figure 11: *Bifidobacterium* Fecal Counts. There was a significant difference with respect to time within the probiotic group ($\alpha \leq 0.10$; P =0.10) and a significant difference between factors of capsule type (Probiotic versus placebo control group) ($\alpha \leq 0.10$; P =0.08)

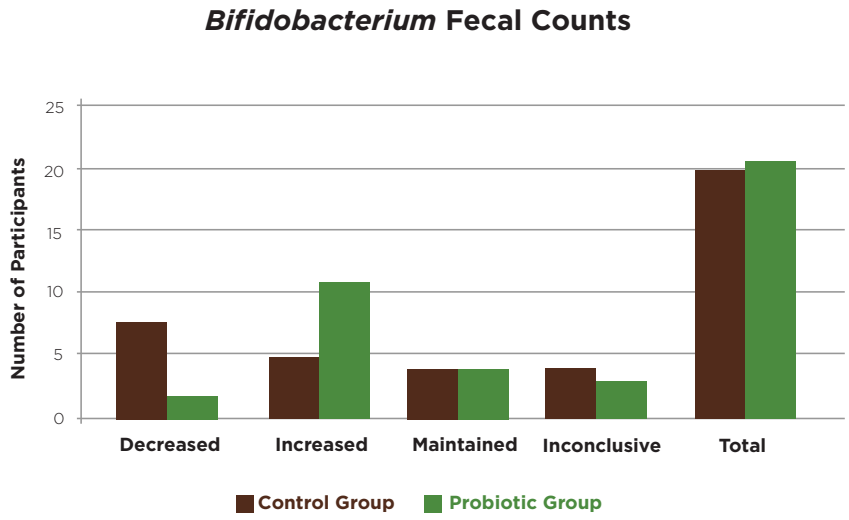
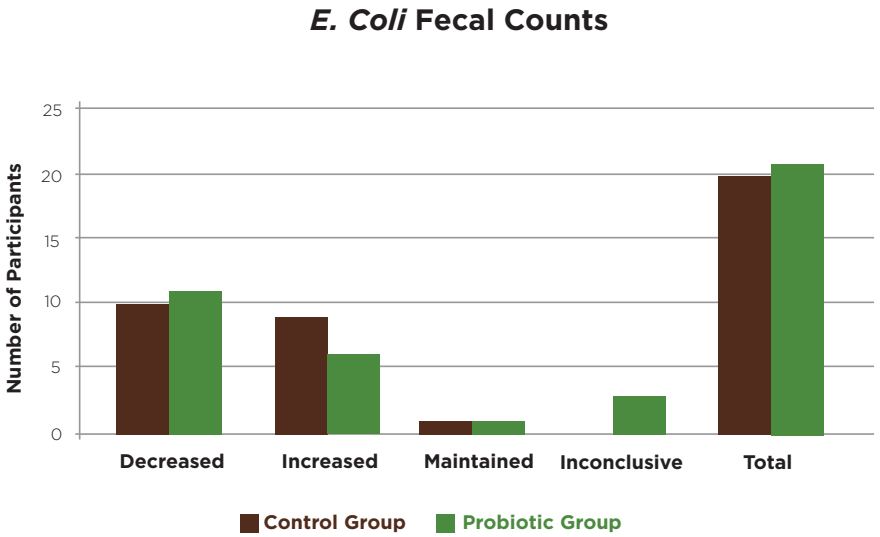


Figure 12: *E.coli* Fecal Counts. Subjects who were administered the placebo demonstrated a numerical increase in levels of *E.coli* while those who were administered the Probiotic experienced a slight decrease in *E.coli*



APPENDIX A

INFORMED CONSENT WAIVER PRESENTED TO AND SIGNED BY ALL PARTICIPATING SUBJECTS

Tolerance and Effectiveness of a Probiotic (Versus Placebo) Delivered in Capsule Form Instead of in Food

I have been informed that the purpose of this research is to study the tolerance and effectiveness of a specific probiotic in capsule form versus the fermented food(s) in which it is typically consumed. A probiotic is a bacteria or mix of bacteria that may be naturally found in the human gastrointestinal system, usually in the large intestine (also known as the colon). Probiotics are used to make or are added to common foods such as yogurt. Eating probiotics can help increase the population of good (non-pathogenic) bacteria making it harder for bad (pathogenic) bacteria to establish colonies in the colon. My participation in this study will involve five trips to the Health Science Center (HSC) on the UW-L campus as follows:

1. 1st trip: I will review and discuss this informed consent with the researchers and if I give informed consent to participate in the study, I will fill out a brief health history and gastrointestinal symptoms questionnaire, after which, I will be informed if I will or will not be eligible to participate in the rest of the study. If eligible, I will sign up for additional days.
2. 2nd trip: I will arrive fasted (having not consumed anything other than water) and provide a 15 milliliter (about 3 teaspoons) blood sample drawn from a vein in my arm by an experienced technician (phlebotomist). I will be given instructions and a container to collect a stool sample. I will also be given a snack to eat before I leave.
3. 3rd trip (soon after the 2nd trip) I will bring my stool sample in the container the researchers provided and I will be given a 30 day supply of probiotic or placebo capsules. I will not be told which I was given until the study is over. I will be instructed how and when to take the capsules over the next 30 days. I will also a diet (food and drink) record packet to complete daily as instructed.
4. 4th trip: (approximately 30 days after the 3rd trip) I will arrive fasted (having not consumed anything other than water) and provide a 15 milliliter (about 3 teaspoons) blood sample drawn from a vein in my arm by an experienced technician (phlebotomist). I will be given instructions and a container to collect a stool sample. I will turn in my diet record and complete another gastrointestinal symptom questionnaire at this visit.
5. 5th trip: I will bring my stool sample in the container the researchers provided and I may accept their gift ($100 gift card) of appreciation to me for my participation.

I realize that my participation in this study is voluntary though if I the study, I may accept a gift of appreciation for my participation. I may also learn something about the effectiveness and tolerance of a dietary supplement after the conclusion of the study. The results of this study may be published in scientific journals or presented at professional meetings. However, no personal information about me will be linked to my data and data will be presented in group form only. I realize that I may withdraw from this study at any time, for any reason. I realize that the researchers want me to contact them by email or phone with any questions or concerns I have before, during, and after the study.

Should an adverse reaction to the supplement (probiotic or placebo) occur, it should be reported immediately to the researcher and a medical professional at the Health Science Center will be consulted if necessary, or, if severe, emergency (911) care should be requested. In the unlikely event that any injury or illness occurs as a result of this research, the Board of Regents of the University of Wisconsin System, and the University of Wisconsin-La Crosse, their officers, agents and employees, do not automatically provide reimbursement for medical care or other compensation. Payment for treatment of any injury or illness must be provided by the subject or subject's third party payer, such as health insurer or Medicare. If an injury or illness occurs in the course of research, or for more information, I should notify the investigator in charge. I have been informed that I am not waiving any rights that may have for injury resulting from negligence of any person or the institution.

Questions regarding the study procedures may be directed to Peg Maher, PhD, RD (608-785-6967 or 608-498-1542) Department of Biology, 1725 State St, La Crosse, WI 54601. Questions regarding the protection of human subjects may be addressed to irb@uwlax.edu.

Participant's Signature: _____ Date: _____

Researcher's Signature: _____ Date: _____

APPENDIX B

GASTROINTESTINAL SYMPTOM QUESTIONNAIRE PRESENTED TO AND COMPLETED BY ALL SUBJECTS PRIOR TO THEIR FIRST AND AFTER THEIR LAST CAPSULE CONSUMPTION

Question	1	2	3	4	5
Have you been bothered by pain or discomfort in the upper abdomen or the pit of the stomach during the last 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
Have you been bothered by nausea during the past 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
Have you been bothered by rumbling in your stomach during the past 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
Has your stomach felt bloated during the past 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
Have you been bothered by diarrhea during the past 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
When going on the toilet, have you had the sensation of not completely emptying your bowels during the past 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
Have you been bothered by hunger pains during the last 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
Have you been bothered by low energy level during the past 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
Have you been bothered by headaches during the past 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
Have you had food cravings in the last 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
Have you had a loss of appetite during the past 4 weeks?	None of the time	Less than 7 days	7-14 days	More than 14 days	All of the time
Overall, How is your health?	Excellent	Good	Fair	Poor	Terrible
How much physical pain have you had during the past 4 weeks?	None	A Little	Some	A good deal	Very much
I am comfortable	Strongly disagree	Somewhat disagree	Neither agree nor disagree	Somewhat agree	Strongly agree
I am as healthy as anybody I know	Strongly disagree	Somewhat disagree	Neither agree nor disagree	Somewhat agree	Strongly agree

APPENDIX C

BRISTOL STOOL CHART

Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on the surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. **Entirely Liquid**

APPENDIX D

SELECTIVE MEDIA AND CULTURE CONDITIONS FOR BACTERIAL STRAINS

Bacillus subtilis
Agar Name: *Bacillus cereus* agar base (Catalog #: 7442, Lot#: 106685)
Company: Neogen Corporation, Accumedia, Lansing, MI
Agar Ingredients: 41 g *B. cereus* agar, 950 mL DIH_2O, after autoclaving: 50 mL egg yolk emulsion and 10 mL polymyxin B
Incubation: Aerobic conditions, 37°C overnight (12-24 hrs)
Selective for: *Bacillus cereus* (blue colonies with halo) *and Bacillus subtilis* (cream to yellow colonies)

Escherichia coli
Agar Name: MacConkey Agar (Catalog #: C6132, Lot#: 12332)
Company: Hardy Diagnostics, Criterion, Santa Maria, CA
Agar Ingredients: 50 g MacConkey agar, 1L DIH_2O, autoclave
Incubation: Aerobic conditions, 37°C overnight (12-24 hrs)
Selective for: Gram-negative bacteria, lactose fermenters (i.e. *Escherichia, Klebsiella, Enterobacter, Hafnia, and Citrobacter*) appear pink, non-lactose fermenters (i.e. *Salmonellal*) appear colorless.

Lactobacillus acidophilus
Agar Name: Rogosa SL Agar (Product#: R1148)
Company: Sigma-Aldrich, St. Louis, MO
Agar Ingredients: 75 g Rogosa agar, 1L DIH_2O, 1.33 mL glacial acetic acid, DO NOT AUTOCLAVE
Incubation: Anaerobic chamber with GasPak™EZ, 37°C C 2-3 days (48-72 hrs)
* GasPak™EZ Anaerobe Container System Sachets with Indicator (BD, Catalog#: 26001)
Selective for: Lactobacillus species appear white to cream in color

Bifidobacterium longum
Agar Name: Liver Veal Agar 500G (Catalog#: 259100)
Company: Becton Dickinson & Company (BD), Franklin Lakes, NJ
Agar Ingredients: 97 g liver veal agar, 10 g lactose, 5 g sodium propionate, 500 mg lithium chloride, 400 mg L-cysteine, 20 mg sodium lauryl sulfate, 1L DIH_2O, autoclave
Incubation: Anaerobic chamber with GasPak™ EZ, 37°C C 2-3 days (48-72 hrs)
*GasPak™EZ Anaerobe Container System Sachets with Indicator (BD, Catalog#: 26001)
Selective for: Anaerobic bacteria (i.e. *Bifidobacterium*) appear white to cream colored and Fastidious aerobic pathogens (i.e. *Neisseria meningitides)*

Candida albicans
Agar Name: DRBC agar (Catalog#: 7591, Lot#: 106023)
Company: Neogen Corporation, Accumedia, Lansing, MI
Agar Ingredients: 31.6 g DRBC agar, 1L DIH_2O, autoclave
Incubation: Aerobic conditions, 25°C 2-7 days (approximately 96 hrs)
Selective for: Yeast (i.e. *Candida*) appear pink

APPENDIX E

LITERATURE REVIEW

Gastrointestinal System

The gastrointestinal (GI or digestive) tract contains a series of hollow organs responsible for nutrient digestion, utilization, and absorption. The mouth, pharynx, esophagus, stomach, small intestine, large intestine (colon), rectum and anus are the specialized organs of the GI tract. A food mass moving through the GI tract is initially called a bolus, after mixing with gastric juices it is called chyme, and then finally, what is left after movement through the colon is referred to as feces. Along the GI tract route nutrients in food, but also bacteria in food, will be subject to neutral and acidic conditions and various digestive enzymes.

The large intestine consists of the cecum and ascending, transverse, descending and sigmoid portions of the colon and is a major site of salts and water absorption and reabsorption. The large intestine is also the most prominent portion of the gastrointestinal system for bacterial colonization with 500 different species of bacteria, and 10^{11} cells/g in the cecum (Bengmark 1998 and Neish 2009). The microbiota are often referred to as "the forgotten organ" due to the diverse beneficial roles of microbes in fiber digestion, vitamin production, inhibition of pathogenic colonization, and immune function (Neish 2009 and Johnstone et al 2014).

Human Gut Microbiota

In the womb, the human gut is completely sterile and immediately colonized after birth (Neish 2009). Microbiome composition not only varies from person to person but it also varies throughout one's lifetime depending on genetics, ethnicity, age, weight, health, medication use, etc (Cani and Delzenne 2009 and Marco and Tachon 2013). The microflora that reside within the human gut generally fall into three different relationship categories: symbiotic (+/+), commensalism (+/o or o/o), and pathogenic (+/-) (Hooper 2001 and Neish 2009). Symbiosis and commensalism that occur between the host and the microorganism is poorly understood and defined. For the purposes of this review, these relationships will be used interchangeably.

Bacterial species within the three portions of the large intestine differs due to varying conditions and nutrient availability. For example, the proximal colon has more abundant bacterial populations due to high substrate availability. In addition, the proximal colon has a more acidic environment, and a more rapid transit than that of the distal colon. The distal colon has a lower concentration of available substrates and a more neutral pH, resulting in slower bacterial growth at this location (Fooks et al. 1999). Most human-endogenous bacterial species are located in the large intestine, are anaerobic in nature and represented by *Bacteroidetes* and *Firmicutes* (Fooks et al. 1999, Ley et al. 2006, Marco & Tachon 2013, Mutlu et al. 2012, and Neish 2009).

Gastrointestinal microbiota flourish and aid in digestion and nutrient absorption by degrading and fermenting various foodstuffs, such as dietary fiber, cellulose, oligosaccharides, proteins, peptides, etc., into short chain fatty acids (SCFAs) (Fooks et al. 1999, Rauch and Lynch 2012, Salminen et al. 1998, and Wong et al. 2006). Prominent SCFA end products include acetate, butyrate, and propionate (Fooks et al. 1999, Rauch and Lynch 2012). The absorption of the produced SCFAs is an efficient process associated with enhanced sodium absorption and bicarbonate excretion (Wong et al. 2006). Acetate is absorbed and transported to the liver to aid primarily in cholesterol synthesis. Propionate, once absorbed, acts as both a substrate and an inhibitor of gluconeogenesis. Butyrate, which is preferentially used over acetate and propionate, plays a role in regulation of cell proliferation and differentiation (Salminen et al. 1998 and Wong et al. 2006). Gut commensals, such as probiotics, exhibit other beneficial effects for the host (Rolfe 2000).

Probiotics

Probiotics are live microorganisms residing in the human gut with low or no pathogenicity and exhibit beneficial effects for the host (Bengmark 1998, Geier et al. 2007, Rauch and Lynch 2012, and Rolfe 2000). Common products containing probiotic bacteria include dietary supplements and foodstuffs such as fermented dairy products, sauerkraut, and salami. Probiotic supplementation has shown positive results for relief of various ailments such as: antibiotic associated diarrhea, constipation, allergies, and diabetes (Al-Salami et al. 2008, Fooks et al. 1999, Goldin and Gorbach 2008, Ranadheera et al. 2009, Rauch and Lynch 2012, and Rolfe 2000). Probiotics have also exhibited protective properties.

The reduction and prevention of pathogenic colonization by *Salmonella typhimurium, Shigella, Clostridium difficile, Campylobacter jejuni, Escherichia coli*, etc. has been a trademark of probiotic supplementation, though the mechanism by which this occurs is poorly understood (Bengmark, 1998). The production of inhibitory substances such as organic acids, hydrogen peroxide and bacteriocins inhibits both gram-positive and gram-negative bacteria. These substances reduce viable cell counts in addition to affecting pathogenic metabolism or toxin production. Viable options for pathogenic inhibition consist of competitive inhibition by blocking adhesion sites or by competing for similar nutrients. Degradation of toxin receptors on the intestinal mucosa may also be a mechanism of action for host protection. Finally, it is thought that probiotics may also play a role in immune system stimulation (indicated for instance, by increased C-reactive protein) (Casula and Cutting 2002, Fooks et al. 1999, Geier et al. 2007, and Rolfe 2000).

Probiotic supplements can contain one or more different bacterial strains that exert different effects on the human gut (Rolfe 2000). Common probiotic strains are lactic acid producers such as *Lactobacillus, Bifidobacterium*, and *Streptococcus* due to their resistance to gastric acids, bile salts, and pancreatic enzymes (Rauch and Lynch 2010, and Rolfe 2000). 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* (Rolfe 2000) (Bengmark 1998).

Not all probiotic supplements are lactic acid producers. *Bacillus subtilis* spores have been used as probiotics, competitive exclusion agents, and prophylactics for human and animal

consumption (Casula and Cutting 2002). *Bacillus subtilis* is a gram-positive, spore forming, rod-shaped bacterium. Gram-positive bacteria contain peptidoglycan in the cell wall, which is responsible for the violet stain (Lim 1998). Under nutrient limiting conditions, *Bacillus* and *Clostridium* can form resistant dormant endospores to environmental stressors and nutrient deprivation, making these bacteria a viable option for a probiotic supplement (Lim 1998). *B. subtilis* have the potential to suppress all aspects of *Escherichia coli* 078:K80 infection in chick models (Casula and Cutting 2002).

The purpose of this study is to determine the tolerance and efficacy of *B. subtilis* as a probiotic supplement. Tolerance will be analyzed through various blood parameters covered in comprehensive metabolic, liver, and lipid panels, in addition to C reactive protein (CRP) levels. Gastrointestinal symptom questionnaires will be filled out by subjects prior to and after capsule consumption, as well as completing daily food diaries and bowel movement records throughout the course of the study. Efficacy will be determined with the use of polymerase chain reaction (PCR) and real-time polymerase chain reaction (qPCR) assays to determine presence and quantity of gut microbes in fecal samples. Fecal smears on microbial specific agar plates will also assist in determining the efficacy of the supplement.