Article

Examining the immunomodulatory effects of the novel probiotic *Bacillus subtilis* DE111

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Abstract: Probiotics make up a large and growing segment of the commercial market of dietary supplements and are touted as offering a variety of human health benefits. Some of the purported positive impacts of probiotics include, but are not limited to, stabilization of the gut microbiota, prevention of gastrointestinal disorders and modulation of the host immune system. Current research suggests that the immunomodulatory effects of probiotics are strain specific and vary in mode of action. Here, we examined the immunomodulatory properties of *Bacillus subtilis* strain DE111 in a healthy human population. In a randomized, double blind, placebo-controlled four-week intervention, we examined peripheral blood mononuclear cells (PBMCs) at basal levels pre- and post-treatment as well as in response to stimulation with bacterial lipopolysaccharide (LPS). We observed an anti-inflammatory effect of *B. subtilis*, manifested as a decrease in immune cell populations within the basal state along with an increase in anti-inflammatory immune cells in response to LPS stimulation. Overall gastrointestinal health, microbiota, and circulating and fecal markers of inflammation and gut barrier function were largely unaffected by DE111 treatment. These data suggest that the novel probiotic *B. subtilis* DE111 may have clinical applications in modulating immune homeostasis via anti-inflammatory mechanisms.

Keywords: *Bacillus subtilis*, flow cytometry, gastrointestinal health, peripheral blood mononuclear cells (PBMC), probiotic, short chain fatty acid

1. Introduction

The human GI tract houses roughly 3.8 X 10^{13} microorganisms comprising up to 1,000 different species (1). These microorganisms function to aid in digestion, protect the host from pathogens, and facilitate development of gut-associated lymphoid tissue (GALT) and other components of the immune system. Several studies have indicated that host-microbe interactions are required for proper GALT development. Germ-free mouse models, mice lacking a gut microbiota, have been shown to have underdeveloped lymphoid organs as well as delayed and decreased somatic diversification of B cells (2). In addition, they have reduced IgA production, decreased T regulatory cells, and impaired gut barrier function (3). Colonization with commensal bacteria ameliorates these deleterious effects in germ-free mice and restores healthy immune function (4). Studies of the interactions between the microbiota and immune cells reveal various immune-modulatory mechanisms, including activation of innate and humoral cell populations, induction of cytokine production, stimulation of secretory IgA production, competitive exclusion of pathogenic bacteria, and generation of bioactive

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metabolites, such as short chain fatty acids (SCFA) (5–8). These interactions have led to the commercialization of probiotic bacteria to promote immune health in consumers.

A novel probiotic bacteria of interest is *Bacillus subtilis* (*B. subtilis*), a rod-shaped, spore-forming, Gram-positive facultative aerobe (9,10). In addition to its activity as a plant growth promoting organism found in the soil, *B. subtilis* is utilized for fermentation in Asian soy-based dishes such as natto and cheonggukjang. *Bacillus subtilis* has recently been considered for its probiotic potential because of its ability to form resistant spores, which enhance the shelf-life of these bacteria as well as their survival in the gastrointestinal (GI) tract. Additionally, recent evidence suggests that *B. subtilis* is part of the normal gut microbiota of humans (11) and may play a critical role in immune development (12) and promotion of GI health (9). It completes its life cycle within the GI tract of humans and other vertebrates, as well as in invertebrate models (11,13). In fact, GI isolates of *B. subtilis* have demonstrated increased sporulation compared to traditional lab-grown isolates of *B. subtilis* (13). It has been hypothesized that, to combat the decreased efficiency of growth in the anaerobic conditions of the GI tract, *B. subtilis* creates an aerobic microenvironment via the production of exopolysaccharide (EPS) biofilms (11). This EPS can also serve as a prebiotic, stimulating growth of other microbes in the GI tract and modulating immune cell populations that reduce intestinal inflammation in mouse models (14).

To further investigate its clinical and commercial applications, we investigated the potential immune modulatory effects of *B. subtilis* strain DE111 (DE111) in a healthy human population. We hypothesized that daily consumption of DE111 for four weeks would modulate the basal host immune status as well as its response to a pro-inflammatory challenge, as determined via alteration in specific immune cell populations. We tested this hypothesis using a parallel arm, double-blind, randomized, placebo-controlled intervention study. Our primary outcomes included flow cytometric analysis of immune cell populations from cultured peripheral blood mononuclear cells (PBMCs), both with and without stimulation by bacterial lipopolysaccharide (LPS). Secondary outcome measures included examining the effects of *B. subtilis* on perceived gastrointestinal health, gut microbiota profiles, and circulating and fecal markers of inflammation and immune function.

2. Results

2.1 Study Recruitment and Compliance

Recruitment and enrollment were conducted under an umbrella protocol for two clinical studies. A total of 93 normal weight to mildly obese healthy male and female adults between 20-62 years old were recruited to the protocol from an initial pool of 153 screened individuals (Figure 1). Of these 93 people, 46 individuals were enrolled into the current study and randomized to either DE111 (n=25) or a placebo control group (n=21). All 21 individuals enrolled in the placebo group completed the study and 23/25 people enrolled in the DE111 group completed the trial, with two individuals lost to follow-up, resulting in a total of 44 people completing the full study protocol.

Total study compliance with the treatment regime was ~96% with individual compliance ranging from 77%-100%. Compliance to specific treatments were as follows: Placebo: average compliance= 97% (range= 77-100%) and *B. subtilis* DE111: average compliance= 96% (range= 83-100%). Additionally, compliance with maintaining diet and body weight was achieved throughout the study. Neither treatment nor diet influenced body weight over the course of the study. The average participant BMI was ~25 kg/m^2, regardless of treatment group, and the average weight fluctuated by <1 kg. There were no differences between groups in blood glucose or other metabolic parameters and no effect of treatment over time (Supplemental Figure 1). Baseline characteristics for participants included in this study are presented in Table 1.

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Table 1: Participant Baseline Characteristics

Treatment	Male	Female	Height (cm)	Weight (kg)	BMI (kg/m²)	Age (yr)
Placebo (n=21)	8	13	169.4 ± 7.3	71.0 ± 10.0	24.7 ± 2.8	34.4 ± 13.0
Bacillus subtilis DE111 (n=25)	10	15	170.8 ± 9.1	72.6 ± 9.3	24.9 ± 2.4	36.9 ± 12.9

Data represents mean ±SD

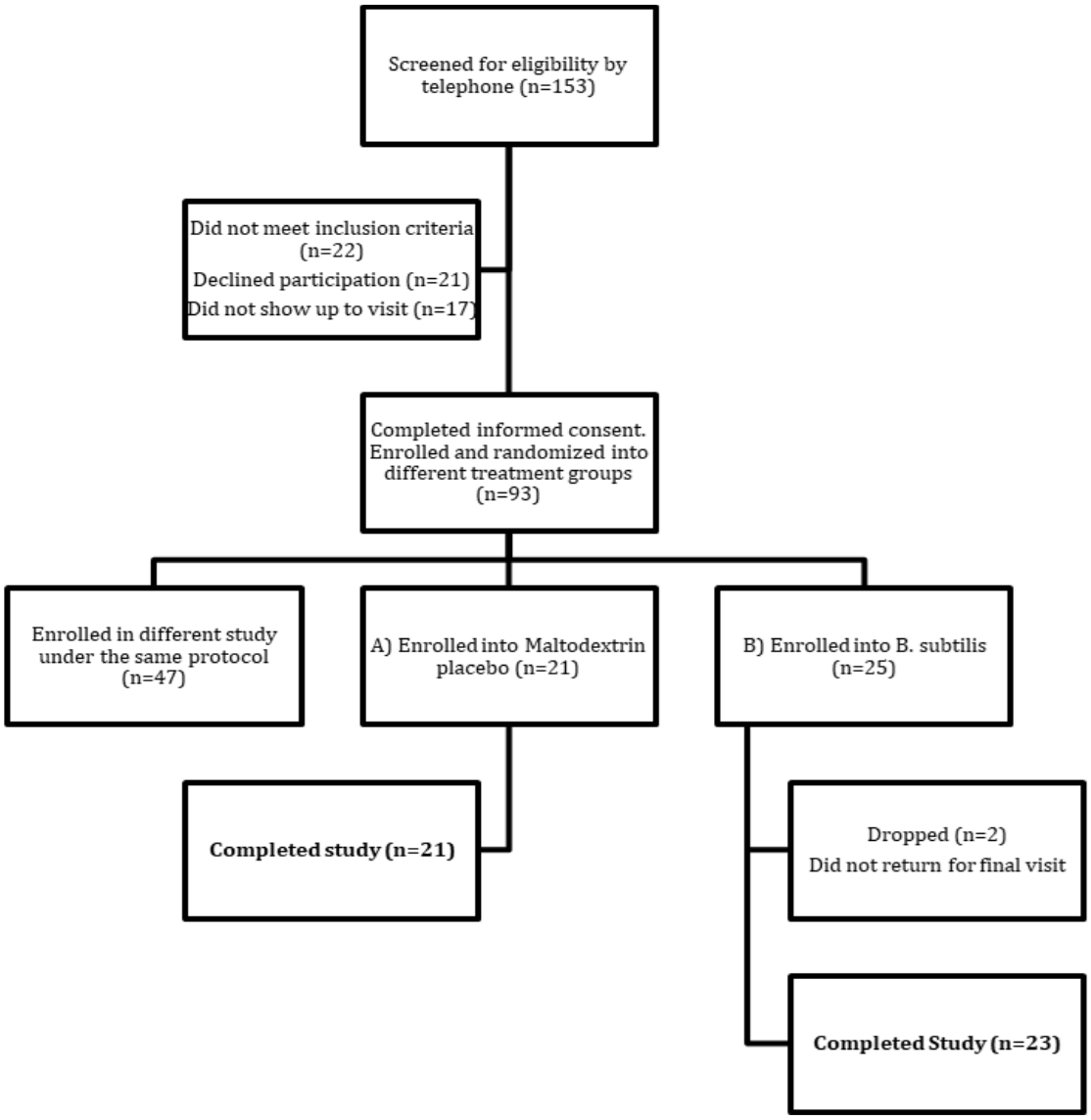

Figure 1. Consort flow diagram of study recruitment, randomization, and completion.

2.2 Dietary Recall

There was poor compliance for completing the self-reported 24-hour food recalls, with only ~54% of participants providing both baseline and four-week dietary intake information. Compliance within each of the treatment groups was as follows: Placebo: average compliance= 48% and *B. subtilis*

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DE111: average compliance= 59%. Based on available data, there was notable variability between individuals, but there were no significant changes in dietary intake of total calories, macronutrients, fiber, cholesterol, or saturated fat from baseline to study completion within either treatment group (Table 2). There were also no significant differences between treatments regarding change in intake over the course of the study.

Table 2: Self-Reported Diet Intake Based on Two 24-Hour Recalls

	Placebo (n=10)		DE111 (n=13)	
	Baseline	**Final**	**Baseline**	**Final**
Energy (kcal)	2269 ± 999	2089 ± 982	2238 ± 741	2389 ± 638
PRO (g)	99 ± 50	96 ± 50	96 ± 40	102 ± 48
TFAT (g)	103 ± 56	86 ± 42	95 ± 40	101 ± 32
SFAT(g)	33 ± 18	27 ± 14	32 ± 19	34 ± 15
CHOL (mg)	358 ± 259	324 ± 271	367 ± 221	439 ± 308
CARB (g)	240 ± 104	225 ± 118	223 ± 79	237 ± 73
FIBER (g)	25 ± 11	22 ± 12	21 ± 6	27 ± 10

Data represents mean ± SD. Abbreviations: PRO (protein), TFAT (total fat), SFAT (saturated fat), CHOL (cholesterol), CARB (carbohydrates)

2.3 *Adverse Events*

A total of seven participants in the *B. subtilis* DE111 group reported adverse events, with the severity of events reported as moderate or unspecified. Adverse events included constipation, flatulence, fatigue, anxiety, and diarrhea. The participant experiencing diarrhea only reported two days of watery stool. The duration of the other adverse events was not reported. One participant on the maltodextrin placebo reported having flatulence and a lack of concentration, not attributed to the study. No adverse events were reported as severe or life threatening, suggesting that there is a low risk associated with DE111 consumption.

2.4 *Perceived Gastrointestinal Health*

Although more participants in the treatment group reported adverse events compared to the placebo group, there were no negative changes in self-reported measures of gastrointestinal health. In fact, there was a significant overall effect for time in symptom severity scores related to gastric function (Figure 2A; p=0.008; CI=0.41, 2.61), suggesting overall improvement in the study population, regardless of treatment group. Although the main effect was driven by decreases in symptom severity scores in both the placebo and DE111 groups, only the DE111 group showed a trending (defined in this study as p=0.51 to 0.08) reduction in severity score from baseline to post-treatment (p=0.056; CI= -3.49, 0.04). Gastrointestinal inflammation, small intestine and pancreas pain, and colon pain scores did not change over time with the treatment or placebo, nor were there any significant differences between the treatment groups (Figure 2).

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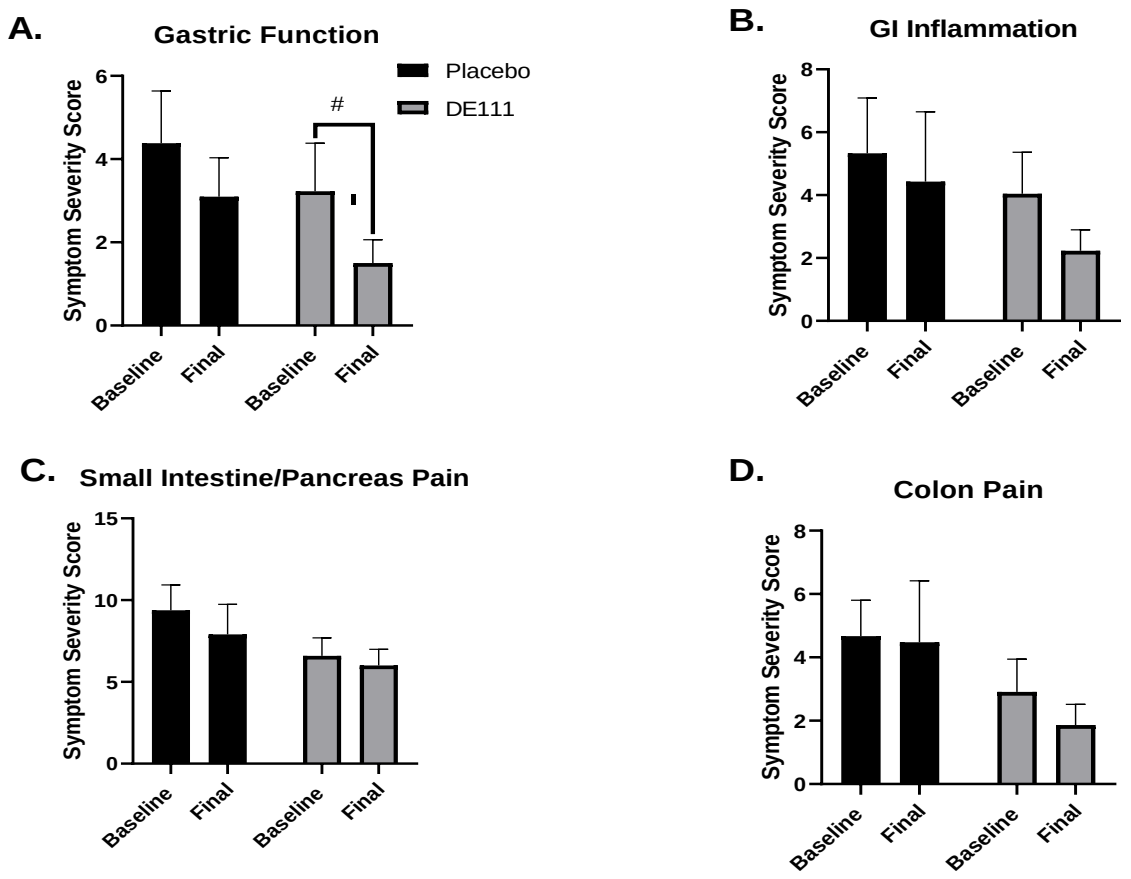

Figure 2. Symptom severity scores were based on answers to a GI symptom questionnaire by Metagenics (15) *that is designed to provide a functional measure of gastric function (A), gastrointestinal inflammation (B), Small intestina/pancreas pain (C), and colon pain (D). Error bars represent ±SEM,# indicates a statistical trend (p=0.051-0.08).*

2.5 Quantitative PCR (qPCR) and 16s Microbiota Analysis

Using qPCR, our 2-way mixed effects model revealed a significant effect for time in *B. subtilis* log DNA amounts detected in stool (p=0.049; CI= -0.61, 0.00). Post-hoc Sidak tests identified that this was driven by an increase in *B. subtilis* DNA in stool samples from individuals in the treatment group (Figure 3A; p=0.044; CI=0.01, 0.96). There were no significant differences between timepoints in the placebo group or between the two treatments. Although the amount of *B. subtilis* DNA was not significantly different between the final timepoint for the treatment and control (p=0.35), the baseline adjusted difference in *B. subtilis* DNA between the two groups was significant using an unpaired t-test (Figure 3B; p=0.01; CI=-0.24, 0.97). Interestingly, *B. subtilis* DNA was detected in most samples, supporting previous reports that these organisms are naturally present in the human gut (11).

Using 16s rRNA sequencing, we next sought to determine whether DE111 consumption altered the community structure of the gut microbiome. We did not detect any significant differences in observed or estimated microbial richness (Supplemental Table 1) or in Bray-Curtis distances between treatment groups or within a given treatment over time (Figure 3C).

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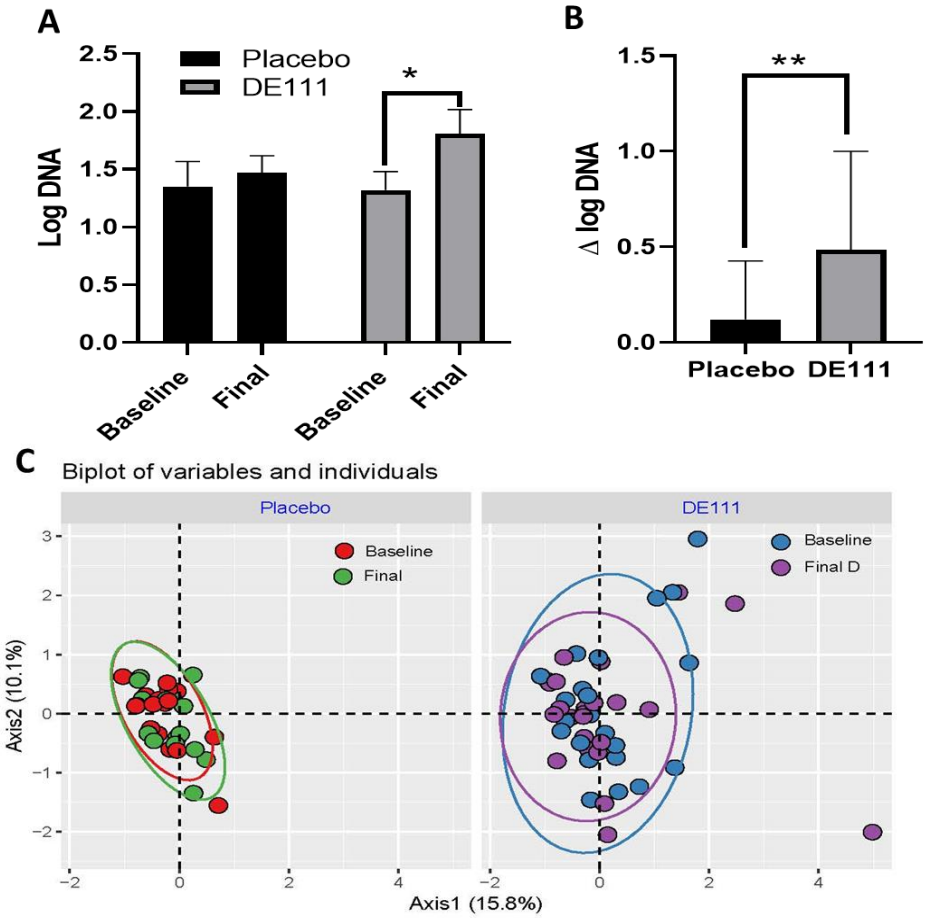

Figure 3. Levels of B. subtilis DNA detected in fecal samples collected pre- and post-intervention (A) and the change in logDNA from baseline between the two intervention groups (B) indicate that DE111 consumption increases B. subtilis in the GI tract. However, four weeks of DE111 consumption did not significantly alter the composition and structure of the gut microbiota (C). Error bars represent ±SEM. * indicates p-values <0.05 and ** indicate p-values <0.01. Ellipses in the PCoA represent the 95% CI.

2.6 Short Chain Fatty Acid Analysis

Using gas chromatography, we measured fecal short chain fatty acids (SCFAs) at both the start and completion of the intervention period. On average, fecal samples contained about 30mM/gram of acetate, and only between 1-2mM/gram of propionate and butyrate. There was a significant interaction effect for acetate (time x treatment; p=0.047; CI=0.04, 4.99), as well as a trending effect for treatment (p=0.062; CI= -3.62, 0.10). This was primarily driven by a significant decrease in acetate in the placebo group over the four week intervention (p=0.046; CI= -3.78, -0.03), resulting in significantly higher levels of acetate in the DE111 compared to the placebo post-intervention (Figure 4A; p=0.017; CI= 0.47, 5.57). There were no significant effects either with treatment, or over time, for propionate or butyrate (Figure 4B, C).

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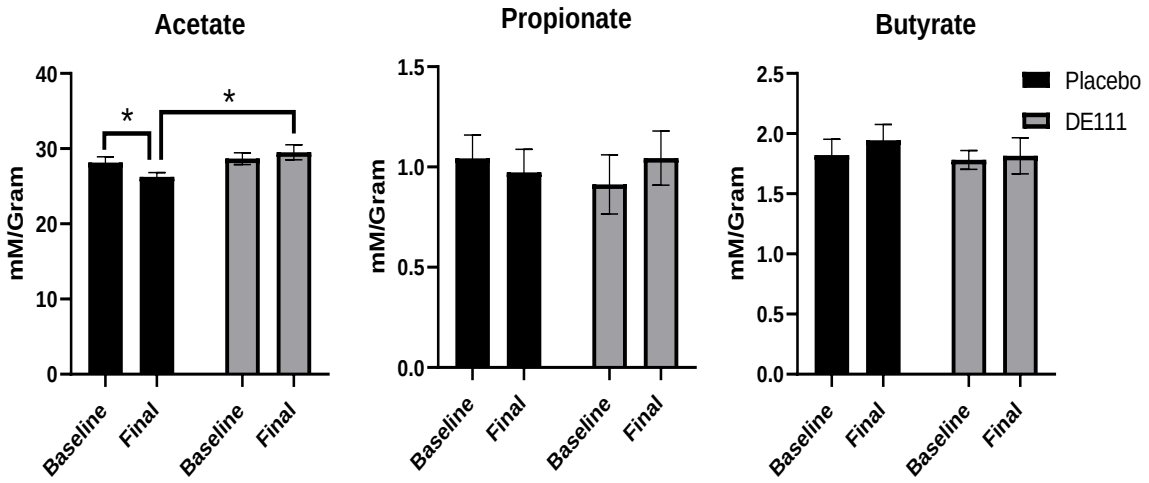

*Figure 4. The major microbially-produced SCFA, acetate (A), propionate (B), and butyrate (C) quantified in feces at baseline and post-intervention. Error bars represent ±SEM, *denotes p<0.05.*

2.7 Immune Cell populations

2.7.1 Basal Cell Counts

Collected PBMC's were cultured and assessed by flow cytometry to determine immune cell populations. There was a significant treatment effect of DE111 over time resulting in significant decreases in CD3+ T cells (Figure 5A; p=0.02; CI=-12111, 1066) and CD25+FoxP3+ T regulatory cells (Figure 5B; p=0.03; CI=-234, 40) when comparing the baseline visit to the final visit. There was also a trending reduction in the DE111 group over time in CD8+ cytotoxic T cells (p=0.07; CI=-3291, 953) and CD25+ T regulatory cells (p=0.06; CI=-75, 21; Figure 5A-B). There was also a trending treatment effect of DE11 resulting in decreased CD4+ T helper cells post-treatment in the DE111 group compared to placebo (p=0.06; CI=-5094, 1378). Both the placebo and DE111 groups showed significant decreases in NKT cells between the baseline and final visits (Figure 5C; p=0.04; CI=-885, 244 and p=0.01; CI=-1084, 25, respectively). There were no significant differences either between or within treatments for myeloid cells or B cells (Figure 5D).

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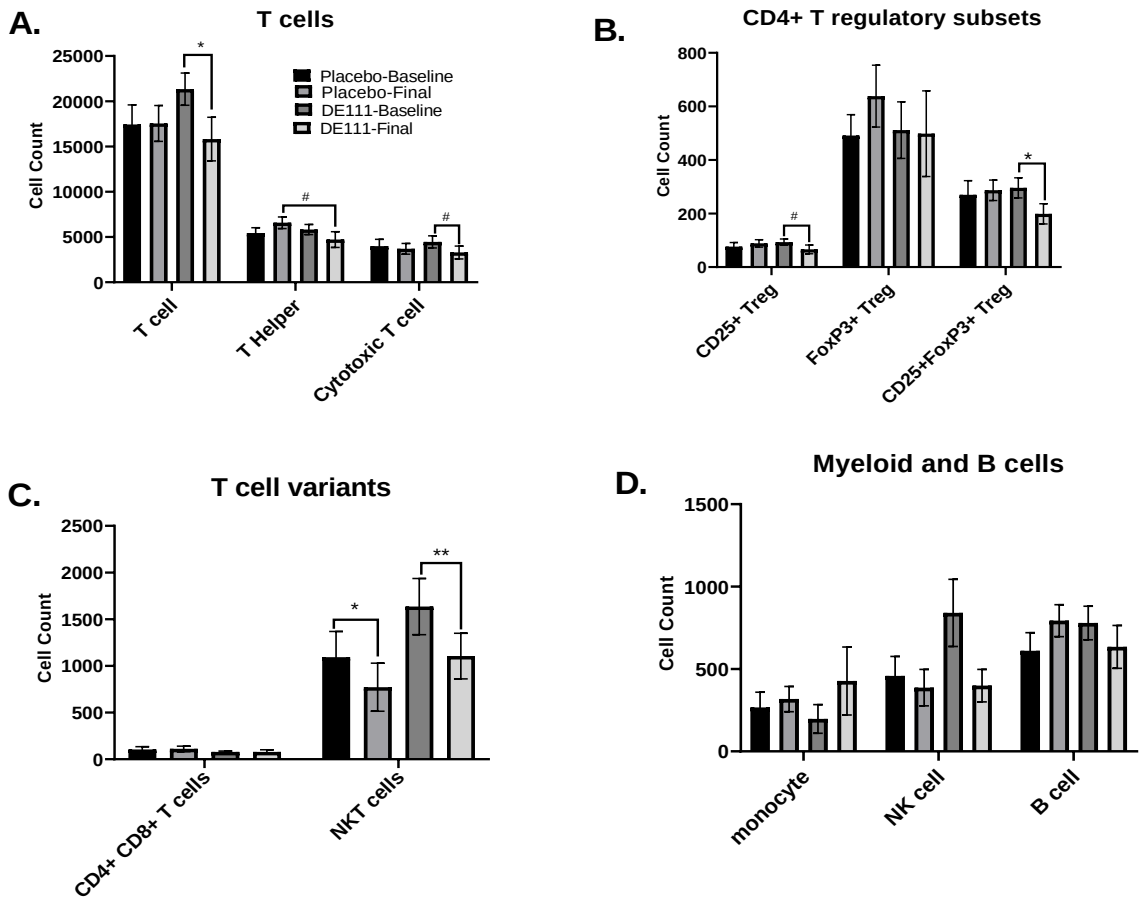

*Figure 5. Basal immune status at baseline and post-treatment in Placebo and DE111 treatment groups: Total CD3+ T cell populations (A), CD4+ T regulatory subpopulations (B), T cell variants (C), and myeloid and B cells (D). Error bars represent ± SEM. * denotes p<0.05, **denotes p<0.01, and # denotes a p value between 0.05-0.08.*

2.7.2 LPS-Stimulated Cell Counts

In order to better understand how DE111 treatment impacted immune cell responses, we stimulated cultured PBMCs with bacterial lipopolysaccharide (LPS). The immune cell response was calculated as a ratio of LPS-stimulated cell counts/basal cell counts. In general, most of the cell counts were not significantly altered with LPS-stimulation (ie. response ratio of ~1; data not shown). However, CD25+ and CD25+FoxP3+ T regulatory cell counts were significantly increased in the DE111 treatment group, both from baseline levels and at the final timepoint between the two treatment groups (Figure 6A,B; Table 3). Similarly, DE111 treatment led to a significant increase in CD4+CD8+ double positive T cells both within and between treatment groups (Figure 6C; Table 3). In contrast, CD8+ cytotoxic T cells were reduced after DE111 treatment compared to the placebo group (Figure 6D, Table 3). There were no significant differences in the percent proliferating cells after LPS stimulation (Supplemental Figure 2).

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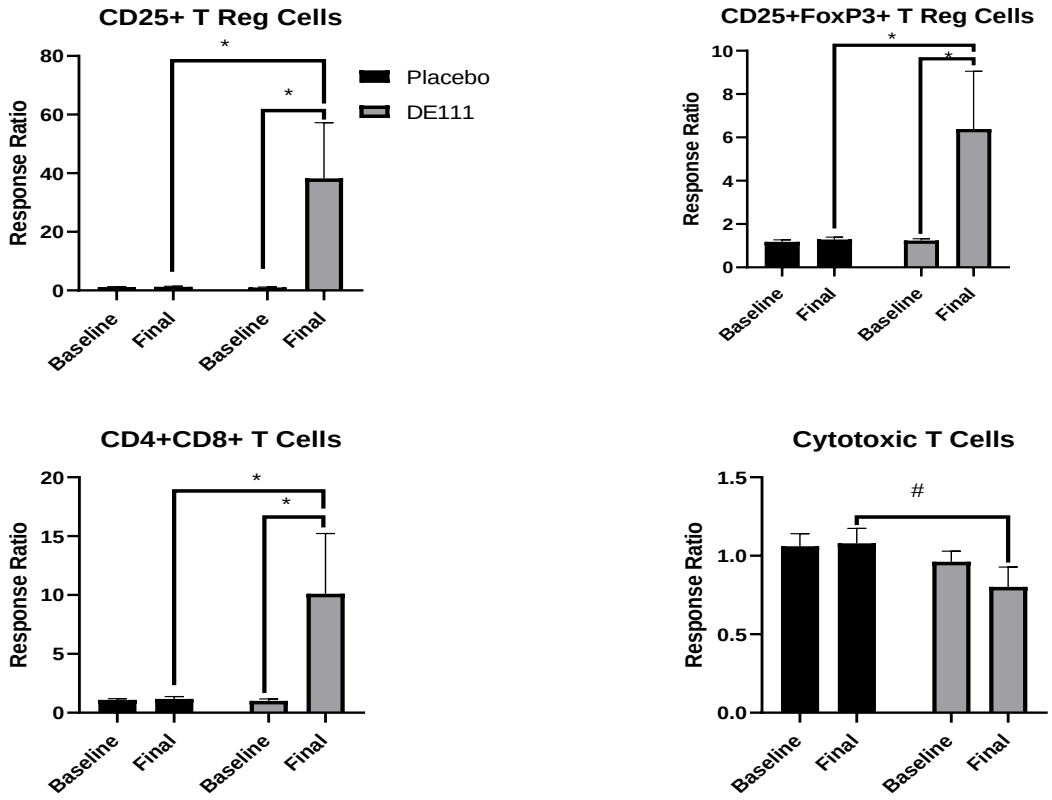

*Figure 6. Cellular responses to LPS stimulation, calculated as stimulated/basal cell counts for CD25+ (A) and CD25+FoxP3+ (B) regulatory T cell populations, as well as double positive CD4+CD8+ T cells (C) and CD8+ cytotoxix T cells (D). Error bars represent ± SEM. * denotes p<0.05 and # denotes a p value between 0.05-0.08.*

Table 3. Statistical analysis (Mixed Effects Model with Sidak post hoc comparisons) for LPS-induced response ratios of significant and trending changes in immune cell populations.

Main Effects	**Time**		**Treatment**		**Interaction**	
Cell Type	**P value**	**95% CI Difference**	**P value**	**95% CI Difference**	**P value**	**95% CI (A1-B1)-(A2-B2)**
CD25+	0.070	-38.80, 1.61	0.073	-38.69,1.80	0.070	-3.27, 77.54
CD25+FoxP3+	0.068	-5.47, 0.21	0.074	-5.42, 0.26	0.080	-0.63, 10.73
CD4+CD8+	0.079	-9.71, 0.55	0.090	-9.55, 0.71	0.084	-1.25, 19.26
CD8+ Cytotoxic	0.435	-0.11, 0.26	0.064	-0.01, 0.39	0.334	-0.55, 0.19
Sidak Post hoc	**DE111 (Baseline-Final)**		**DE111 vs Placebo (Final)**			
Cell Type	**P value**	**95% CI Difference**	**P value**	**95% CI Difference**		
CD25+	0.021	4.99, 69.34	0.022	4.59, 69.43		
CD25+FoxP3+	0.023	0.63, 9.68	0.025	0.55, 9.66		
CD4+CD8+	0.027	0.90, 17.28	0.036	0.49, 17.36		
CD8+ Cytotoxic	0.380	-0.46, 0.14	0.088	-0.59, 0.03		

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2.7 *Inflammation and Gut Barrier Function*

We assayed circulating levels of IL-6 and plasma zonulin to determine basal inflammation and gut permeability before and after the intervention. There were no significant differences either between treatment groups or within treatments over time for any of these parameters. Finally, we also examined sIgA in the stool to assess intestinal immune responses. Again, we observed no differences between treatment groups or within treatments over time for these parameters. (Supplemental Figure 3).

3. Discussion

Spore-forming *Bacillus subtilis* is becoming increasingly popular as a probiotic supplement due to its enhanced shelf-life and survivability in the human digestive tract relative to other bacterial species. Although the *in vivo* viability, safety, and tolerability of several strains of *B. subtilis* have been assessed in appropriate human populations (16–19), little is known about other specific benefits these bacteria may convey. We recently demonstrated that four weeks of *B. subtilis* DE111 supplementation was associated with improved lipid parameters and endothelial function in healthy adults (20). In addition, several *in vitro* and animal model studies have suggested that *B. subtilis* may modulate the mucosal immune system via production of antimicrobial peptides, exopolysaccharides, and quorum sensing molecules, as well as through favorable modifications to the gut microbiota (21–23). In elderly patients, intermittent use of *B. subtilis* strain CU1 increased fecal and salivary sIgA (24). Beyond this, the effects of *B. subtilis* on the human immune system are still largely unexplored, and to our knowledge have not been assessed in an LPS-challenge model.

Here, we observed that oral consumption of *B. subtilis* DE111 for four weeks induced changes in peripheral immune cell populations that are consistent with anti-inflammatory effects. Specifically, we observed a DE111-associated decrease in several T cell subsets, driven by modest reductions in CD4+ helper T cells and CD8+ cytotoxic T cells, and more substantially by decreases in CD25+ and CD25+FoxP3+ Tregs and NKT cells. There have been a few similar studies looking at T cell changes in human PBMCs either after oral probiotic feeding or co-culture of the PBMCs with cell free bacterial supernatants. For example, FoxP3+ Tregs were increased after eight weeks of consuming *Bifidobacterium infantis* (25). In contrast, co-culture of cell free supernatants of *Lactobacillus reuteri* with *Staphylococcus aureus*-stimulated PBMCs had no effect on FoxP3+ Tregs and dampened activation and proliferation of both CD4+ helper T cells and CD8+ cytotoxic T cells (26). In the context of existing literature, our results support the broader hypothesis that probiotic effects on immune function are species-specific and highlight the need for development of standardized assays to assess immune function within human populations.

It is also important to note that most immune cells found in peripheral circulation are naïve lymphocytes and myeloid cells. When myeloid cells activate naïve lymphocytes via antigen presentation and cytokine stimulation, the lymphocytes migrate to the lymph nodes as effector cells. Our observed decreases in these T cell subsets, therefore, could reflect an anti-inflammatory effect of DE111 via decreased cell activation resulting in decreased overall circulating T cells. However, without measurement of specific myeloid and T cell activation markers respectively, these hypotheses are based on speculation.

In addition to assaying basal cell counts before and after probiotic treatment, we also sought to determine how DE111 impacted the immune response to a pro-inflammatory challenge. To test this, we cultured PBMCs with bacterial lipopolysaccharide. We chose to use a chronic (low dose LPS for 72 hours) rather than acute (high dose LPS for shorter duration) stimulation model because several recent studies have indicated that environmental factors such as poor diet and excess body weight lead to a state referred to as “metabolic endotoxemia”, which over time can result in development of several chronic diseases in an otherwise healthy population (27). In the context of this model, we observed DE111-associated increases in CD25+ and CD25+FoxP3+ Tregs, as well as double positive CD4+CD8+ T cells after LPS stimulation. These regulatory T cell subsets act to resolve inflammation and modulate immune responses. While the function of the double positive CD4+CD8+ T cells is still

unclear, they are surmised to play roles in both suppressive and cytotoxic responses (28) and exhibit an increased capacity to produce cytokines and display phenotypic profiles associated with memory T lymphocytes (29). Interestingly, there was a slight decrease in CD8+ cytotoxic T cells. This may be due to the fact that these cells primarily respond to viral pathogens and we challenged PBMCs with a bacterial effector molecule or this could be the result of transcriptional reprograming towards a CD4+CD8+ phenotype (28). Overall, our results indicate an expansion of regulatory immune cells from DE111-treated individuals in response to low-grade, pro-inflammatory stimulation. Therefore, DE111 may be useful in suppressing responses driven by obesity and diet-derived metabolic endotoxemia. This is consistent with our previous observation of improved lipid parameters and endothelial function in DE111 treated individuals, which can result from metabolic dysregulation driven by inflammation (20). Finally, it should be noted that even within the DE111 treatment group, the responses between individuals were highly variable and the observed differences in immune cell populations was largely driven by a group of responders. Therefore, further study into the factors that drive individual responsiveness to DE111 is also warranted.

One limitation to establishing the immunomodulatory effects of *B. subtilis* DE111 in our study is the under-representation of outcomes related to mucosal immune activity. Probiotics largely exert their immunomodulatory effects through direct interactions with immune cells in the intestinal mucosa. The mucosal immune system hosts the vast majority of innate and effector immune cells as well as specialized cells that limit systemic inflammation by acting locally via an array of anti-inflammatory mechanisms (30). However, due to the limitations of obtaining mucosal tissue from healthy human participants, studying PBMCs *in vitro* is a commonly used proxy, particularly when monitoring immune responses to enteric pathogens (31), although it may not accurately reflect DE111's effects on mucosal immunity. To more thoroughly assess mucosal immune responses and gut barrier function, we measured fecal sIgA and plasma zonulin. We also assessed plasma IL-6 levels as a circulating inflammatory marker. However, none of these measures were affected by probiotic treatment. These results are consistent with a study in Division 1 athletes who also consumed *B. subtilis* DE111 at the same dose for 12 weeks and reported no changes in plasma zonulin (17). Unlike the current study, they did report a decrease in a circulating inflammatory molecule (TNF-a) and an increase in sIgA in the DE111 treated group, although this was measured in saliva rather than in feces. These differences may have resulted from the increased duration of probiotic use or because the athletes were all involved in an intense physical training program during the study that likely resulted in elevated basal inflammation. In contrast to these results, Lefevre et al. suggest that intermittent use of *B. subtilis* CU1 did not alter circulating inflammatory markers compared to placebo, although they did report a transient increase in IFN-g from baseline levels after the first cycle of probiotics (24). They also reported elevated fecal and salivary sIgA with probiotic treatment relative to the placebo (24). In a murine model, oral administration of *B. subtilis* spores also increased serum IFN-γ as well as splenic macrophage and natural killer cell activity (32). While we did not measure IFN-γ, it is important to note that our observed decreases in specific immune cell populations in the non-stimulated PBMCs would be consistent with decreased serum IFN-γ, while the expansion of these populations in the LPS-stimulated cells would likely have resulted in increased IFN-γ. Therefore, an understanding of the background inflammatory environment in which these cell population and cytokines are measured will be key in interpreting immunologic responses to probiotics.

The results from our secondary outcome measures, specifically safety and tolerability of *B. subtilis* DE111 intake, as well as its overall impact on microbiota stability, were largely confirmatory. For example, a study that administered five million CFU/day of *B. subtilis* DE111 to 41 healthy adult participants reported after ~20 days of consumption that blood metabolic parameters remained within normal ranges and there were no significant gastrointestinal disturbances or alterations to bowel movements (19). In addition, their reports of increased fecal counts of *B. subtilis* is consistent with the treatment-associated increases that we report in our qPCR data and support that these bacteria do survive intestinal transit (11). A more recently published study examined the impact of *B. subtilis* DE111 on the gut microbiota of preschool aged children after an 8-week consumption period (18). They concluded that DE111 did not disrupt the global microbiota community structure,

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as evidenced by a lack of differences pre- and post-treatment in b-diversity (determined using Bray-Curtis dissimilarity), which is consistent with our findings. They also did not see pre- and post-treatment changes in taxa richness, although diversity did increase with treatment. While we did not see concurrent changes in bacterial diversity, this may be due to the differences in age of the study population. Their study focused on children, who have a much more dynamic microbiota than adults. However, the results of our study, in the context of the existing literature, suggest that DE111 is safe, tolerable, and while it may alter specific taxa, it does not disrupt the general homeostasis of the gut microbiota.

A limitation of the current study was the use of cryopreserved PBMCs for assessing immune cell populations. Freshly isolated cells have been shown to respond differently to LPS-stimulation than cells that have been frozen and thawed prior to culturing and experimentation (33,34). However, due to the duration of the study and the need to minimize variability during staining and flow cytometric analysis, it was most feasible to freeze cells and process them as large cohorts after completion of the clinical trial. Despite this limitation, our study demonstrated that consumption of *B. subtilis* DE111 displayed immunomodulatory and anti-inflammatory effects on several T cell subsets. This is supported by the observed reduction in immune cell populations within the basal state, along with expansion of regulatory immune cells in response to LPS challenge. To further elucidate *B. subtilis* DE111 interactions and mode of immune modulation within the gut, direct stimulation of immune cells in mucosal tissue should be explored. These findings show preliminary yet promising results for utilizing DE111 as an immune-modulating probiotic supplement in a healthy adult population.

4. Materials and Methods

4.1 Study Design. This study was conducted as a parallel-arm, randomized, double-blind, placebo-controlled, four-week intervention trial in healthy adults. Participant eligibility was determined by telephone interview, and subjects arrived at the Food and Nutrition Clinical Research Laboratory (FNCRL) at Colorado State University, to provide written consent. Eligibility was confirmed through anthropometric measurements of height and weight to determine BMI. After BMI was confirmed, participants were randomly assigned to 1 of 2 coded treatments of maltodextrin placebo (n=21) or Bacillus subtilis strain DE111 spores (1x109 CFU; Deerland Probiotics & Enzymes, Kennesaw, GA) (n=25). Over the course of the 4-week intervention, participants were asked to consume one 15 mg capsule per day containing the dispensed treatment.

4.2 Participant Characteristics. Ninety-three healthy male and female adults aged 20 to 62 years of age with a BMI of 20 to 34.9 kg/m2 were recruited into the study (Figure 1). Participants were recruited from the Fort Collins, CO area by email, social media platforms, fliers, and word of mouth. Participants were prescreened for eligibility through a phone interview. Those who met the inclusion criteria were scheduled for an onsite screening visit to confirm eligibility. Exclusion criteria included the following: A) those under 18 or over 65 years of age; B) BMI outside the range of 20-34.9 kg/m2); C) pregnancy or breastfeeding; C) current prescription medication use; and D) use of antibiotics in the last 2 months. Qualified participants were asked to maintain their regular exercise and dietary habits throughout the study, abstain from supplemental pre- or probiotics, and to limit alcohol consumption to one drink per day for women and two drinks per day for men, or no more than seven drinks per week. Participants were randomized and enrolled into different treatment groups, forty-seven of which were enrolled into a separate study under the same protocol (Protocol #19-9145H). Of the total enrolled participants (n=94), 44 completed the currently outlined study.

Participants were asked to fast for eight hours (including no caffeine, soda, tea, etc.) and abstain from exercise for 12 hours prior to arrival in the clinic. Additionally, participants were asked to delay consumption of any medication or dietary supplements for 24 hours prior to the study visit. Participants arrived at the FNCRL for their baseline visit to undergo sample collections (blood and stool) and analysis procedures (weight/height, blood pressure, pulse wave and endothelial function analysis, gastrointestinal symptom screening, and medical health history questionnaire) as previously described (20). Whole blood was collected from the antecubital vein and analyzed for metabolic and lipid parameters using a Piccolo Xpress (Abaxis; Union City, CA) with Piccolo Metlyte

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plus CRP and Lipid Panel reagent discs. The methods and results for blood pressure and endothelial function are published elsewhere (20). Stool samples were collected by participants in study-provided collection containers and stored under refrigeration prior to being returned to the clinic. Aliquots were removed for DNA and short chain fatty acid (SCFA) extraction and stored at -80°C until processed.

Over the duration of the intervention period, participants were asked to maintain their regular diet and exercise regime, and record bowel movements in a daily log using a Bristol stool chart. In addition, they were asked to complete two 24-hr dietary recalls (1 weekday and 1 weekend day), prior to each of their clinic visits, via computer software Automated Self-Administered 24-hour dietary assessment tool (ASA24) developed by the National Institutes of Health, National Cancer Institute. At the end of the 4-week treatment period, these analyses and sample collections were conducted again at the final visit. Participants were asked to return any unused treatment capsules to establish treatment compliance. All participants provided informed written consent prior to participating in the study, which was conducted in accordance with the Declaration of Helsinki and approved by the Colorado State University Institutional Review Board for Human Subjects (Protocol #19-9145H).

4.3 Isolation of Peripheral Blood Mononuclear Cells. Ten mL of whole blood was collected from antecubital veins into ethylenediaminetetraacetic acid (EDTA) treated vacutainer tubes. PBMCs were isolated from whole blood within six hours of collection via density gradient centrifugation and a series of washes. Initially, whole blood was diluted with 1X Phosphate Buffer Solution (PBS) + 2% Fetal Bovine Serum (FBS) (Atlas Biologics; Fort Collins, CO) at a 1:1 ratio and transferred into 50 mL SepMate™ tubes containing 17 mL of density gradient medium, Lymphoprep (Stemcell Technologies Inc; Vancouver, Canada). Tubes were centrifuged for 10 min at 1,200xg at room temperature. The separated plasma and PBMCs were poured off and diluted with an equivalent volume of 1X PBS + 2% FBS and centrifuged at 300xg for 8 min. Tubes were decanted and pelleted cells were resuspended in an equivalent volume of 1X PBS + 2% FBS for a final wash and centrifugation. Cells were counted using a Cellometer Auto T4 Cell Counter (Nexcelom Bioscience LLC; Lawrence, MA) to calculate the appropriate volume of cell freezing media, CryoStor (Biolife Solutions; Bothwell, WA). Pelleted cells were re-suspended in CryoStor and placed in Mr. Frosty Containers at -80°C for 12-24 hours before final storage in liquid nitrogen.

4.4 PBMC Culture and Stimulation. All cell processing was performed aseptically in a laminar flow hood. A complete culture medium containing 1X RPMI-1640 (Corning; Corning, NY), 10% FBS (Atlas Biologics; Fort Collins, CO), and 1% penicillin/streptomycin [100 U/mL penicillin and 100 µg/mL streptomycin] (HyClone; Tianjin, China) was warmed to 37°C in a water bath. Frozen human PBMCs were rapidly thawed in a 37°C water bath. Samples were then transferred to a 15mL conical tube and warm media was added slowly at a rate of 1 mL/5 sec to a final volume of 10mL. Cells were then centrifuged for 8 min at 300xg at 25°C and decanted, followed by two additional washes. Finally, cells were plated for an overnight recovery period at about 1-2x10^6 cells/mL.

After the 24-hour rest, cells were counted and seeded at a desired concentration between 2-5x10^5 cells/well in a U-bottom, 96-well, untreated plate to prevent cell adherence. All samples were plated in duplicate. Appropriate wells were then labeled with proliferation marker, CFSE (Biolegend; San Diego, CA) according to the manufacturer's instructions for a final concentration of 1 µM. Finally, designated wells were spiked with *E. coli* LPS (Sigma-Aldrich; St. Louis, MO) to achieve a final concentration of 1 µg/mL. Cells were incubated at 37°C and 5% CO2 for 72 hours. Post incubation, sample plates were centrifuged for 5 min at 400xg at 4°C and pelleted cells were stained for flow cytometry.

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4.5 Flow Cytometric Analysis. PBMCs were washed with 1X PBS, centrifuged (500 g, 4 min, 4°C), resuspended in LIVE/DEAD fixable Yellow (Invitrogen) and incubated on ice for 30 min. Cells were then pelleted, washed, resuspended in Human TruStain FcX Receptor blocking solution (Biolegend, San Diego, Calif., USA) and incubated on ice for 10 min. After blocking, cells were surface stained with the following fluorescently conjugated antibodies: APC-anti-CD25 (clone M-A251), BV570-anti-CD3 (clone UCHT1), Pac Blue-anti-CD4 (clone SK3), BV750-anti-CD8 (clone SK1), BV605-anti-CD19 (clone HIB19), AF700-anti-CD14 (clone HCD14), BV785-anti-CD16 (clone 3G8), PE-anti-CD56 (clone 5.1H11), and incubated on ice for 30 min. Super Bright Complete Staining Buffer (eBioscience, San Diego, Calif., USA) was used per the manufactures instructions in stain master mixes which contained more than one polymer dye. Cells were then processed for nuclear staining using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufactures instructions and stained with BV421-anti-FoxP3 (clone 206D). All antibodies were purchased from Biolegend (Biolegend; San Diego, CA). Samples were analyzed using a Cytek Biosciences Aurora 4 laser spectral flow cytometer (Cytek Biosciences; Fremont, Calif) and acquired data was analyzed using FlowJo™ ver 10.6.1 Software (Becton Dickinson, Franklin Lakes, NJ).

4.6 ELISA Assays. Plasma collected from whole blood was used to measure circulating IL-6 (Invitrogen, Carlsbad, CA) and zonulin (Elabscience, Houston, TX), and fecal samples were assayed for secretory IgA (BioVendor LLC, Asheville, NC) by ELISA. All kits were run according to manufacturer's instructions. Target analytes were quantified by fitting to standard curves provided with each kit.

4.7 Fecal DNA Extraction and Analysis. Fecal DNA was extracted from samples using the FastDNA Extraction Kit (MP Biomedical) and used for qPCR and preparation of 16s sequencing libraries. Quantitatiive PCR was conducted with *B. subtilis*-specific primers (Forward: 5'-AAGTCGAGCGGACAGATGG-3'; Reverse: 5'-CCAGTTTCCAATGACCCTCCCC-3') on a CFX96 Touch Thermocycler (Bio-Rad Laboratories, Hercules, CA) using the following amplification program: 95°C for 3 min followed by 30 cycles of 95°C for 15s, 60°C for 30s, 72°C for 30s with a final extension 72°C for 5 min. *Bacillus subtilis* levels in samples was quantified by generation of standard curves using purified B. subtilis DNA. Sequencing libraries were prepared following the Earth Microbiome Project protocols as previously described (15). Amplicon libraries were sequenced on an Illumina Miseq at the Next Generation Sequencing Core at Colorado State University. All sequence data was processed using the DADA2 pipeline in QIIME 2 ver 2020.8.0. Alpha diversity parameters were calculated in QIIME 2 and MyPhyloDB ver 1.2.1 was used for calculating Bray-Curtis distances and PCoA visualizations.

4.8 SCFA Extraction and Analysis. SCFA acids were extracted from fecal samples and quantified by Gas Chromatograph with Flame Ionization Detection (GC-FID) as previously described (35). Briefly, one gram of feces was sonicated in acidified, HPLC-grade water (pH brought to 2.5 with HCl) and allowed to sit at RT for 10 min. Samples were then centrifuged, and supernatants removed and frozen overnight at -80°C. The supernatants were then thawed, centrifuged again to sediment any remaining particulate matter, and filtered through 0.45 micron filters. Collected supernatant was analyzed on a GC-FID (Agilent 6890 Plus GC Series, Agilent 7683 Injector Series, GC Column: TG-WAXMS A 30 m × 0.25 mm × 0.25 μm). Peak areas were normalized to the internal standard and quantified using standard curves from dilutions of commercial stocks.

4.9 Statistical Analysis. R software ver 3.6.1 and Graphpad Prism ver 8.3.0 were used for all statistical analyses. Linear mixed effects model (time x treatment) with post hoc Sidak's multiple comparison test was used for all analyses. Clinical data was evaluated for assumption of normality and equal variance. Incomplete sample sets with missing baseline and/or final blood samples were excluded from the immune cell counts (analyzed: n=17 for placebo group, n=18 for DE111), but were included in all other analyses. Microbiome alpha-diversity comparisons were done using non-

parametric Kruskil-Wallis tests and Bray-Curtis distances were evaluated for significance by PERMANOVA with 1000 iterations.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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Abbreviations

CFU	Colony forming units
GI	Gastrointestinal
FNCRL	Food and Nutrition Clinical Research Laboratory
PBMC	Peripheral blood mononuclear cell
SCFA	Short chain fatty acid

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