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Probiotic intervention not beneficial to prevent antibiotic-associated diarrhea in absence of antibiotic-induced microbiome disruption

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Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. One of the most common uses of probiotics is for prevention of antibiotic-associated diarrhea (AAD). Here we report on the PLAY-ON study (Probiotics: Live and Active Yogurt Cultures for Healthy Children ON Antibiotics) which was a randomized, placebo-controlled, double-blinded study in healthy children who were prescribed antibiotics for acute outpatient upper respiratory illnesses, such as bacterial sinusitis or streptococcal pharyngitis. The treating clinician determined the antibiotic, its dosage and duration. Participants received either a control yogurt or a yogurt containing the probiotic, *Bifidobacterium animalis* subsp. *lactis*BB-12. Our primary aim was to determine whether the probiotic prevented AAD. The microbiome of participants was examined longitudinally. Diarrhea rates were nearly identical at 2% (3 cases in the active group, 2 cases in the control group); adverse events were also similar between groups. Thus, our study did not find any differences in the incidence of AAD between those who took the probiotic and the control group. The microbiota of both groups returned to baseline by day 14 and assessment of the microbiome showed no difference in levels or types of antibiotic resistance genes. Broad-spectrum antibiotics are associated with higher rates of adverse events, including AAD, than narrow-spectrum antibiotics, as observed in children taking antibiotics for respiratory tract infections[3,4].However, if the antibiotic does not significantly disturb the microbiota there may be no discernable role for the probiotic. This hypothesis is supported by the participants who used broad spectrum penicillins had a significantly higher rate of diarrhea compared to narrow spectrum antibiotic users, 9.1% and 0.51% respectively, (p=0.004)This study suggests that prescribing short-course, narrow spectrum antibiotics can reduce microbiome disruptions, concomitant AAD, and the need for probiotic intervention. . The antibiotics used in our study did not disrupt the microbiome to the extent expected, which likely accounted for the low observed incidence of AAD. Importantly, this observation can inform clinicians' choice of antibiotic, steering them toward equally therapeutic antibiotics that are less disruptive to the microbiota and better tolerated. Although probiotics are often recommended to prevent AAD, our study demonstrates that in the absence of microbiome disruption, they may not provide benefit.

Abbreviations

AAD	Antibiotic-associated diarrhea
AEs	Adverse events

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ARGs	Antibiotic resistance genes
BB-12	*Bifidobacterium lactis* subsp. *lactis* BB-12
CARD	comprehensive antibiotic resistance database
PCoA	Principal coordinates analysis
PERMANOVA	permutational multivariate analysis of variance
RPKM	Reads per kilobase of genome per million bases of recruited sequences

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Introduction

Several different probiotics - defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (1) - have been shown to reduce the risk of developing antibiotic-associated diarrhea (AAD)[1–5]. Although the mechanism(s) are not known, the evidence-based use of specifically tested probiotic preparations has been adopted in clinical settings to help mitigate AAD[6]. AAD is more than a bothersome adverse event of antibiotic treatment; it is associated with prescription noncompliance, morbidity and overuse of second-line antibiotics. Children are often placed on antibiotics, and the overall rate of diarrhea associated with antibiotic usage is 20–35%, with 1–2% of all AAD patients testing positive for *Clostridium difficile*[7–15]. AAD is linked to a cascade of several disruptions to normal gut function, including an altered gut microbiota, decrease short chain fatty acid, accumulation of luminal carbohydrates, pH changes, water absorption and, ultimately, diarrhea[16].

We previously showed in a randomized, controlled trial that antibiotic-induced microbiota disruption led to reduced short chain fatty acid levels in fecal samples and that these reductions could be ameliorated by a probiotic. This provided insight into a mechanism for how probiotics might be able to reduce incidence of AAD. Specifically, we observed that amoxicillin-clavulanate treatment suppressed fecal acetate levels in healthy subjects treated or untreated with probiotic yogurt containing *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12)[17,18] On day 30 (a priori chosen end of study), fecal acetate levels in the probiotic-treated subjects returned to the baseline levels whereas the acetate levels in the group not getting the probiotic remained suppressed. Additionally, we found that antibiotic-induced reduction in the Shannon diversity index of the gut microbiota was larger and more sustained in the group not receiving probiotic compared to the probiotic group. Others have postulated that probiotics may attenuate the negative effects of antibiotics on the gut microbiota by reducing luminal pH, producing antipathogenic substances, competing with opportunistic pathogens for nutrients, enhancing mucosal barrier function, among other potential mechanisms[19–24].

BB-12 is a probiotic strain that is widely available in commercial products and has been the subject of more human efficacy trials than any other *Bifidobacterium*strain (Email correspondence with Dr. Mirjana Curic-Bawden, Senior Principal Scientist and Application Manager, Fermented Milk and Probiotics, working for Chr. Hansen)[25]. Our overall goal was to determine the efficacy of BB-12 in preventing AAD in children, and gain insight into the potential underlying mechanisms.

Further, to better understand the potential mechanism, we conducted longitudinal analysis of the fecal microbiota using both 16 S rRNA amplicon sequencing and shotgun metagenomics to evaluate the impact of administration of BB-12 in a pediatric population receiving antibiotics.

Methods

The PLAY-ON study was a randomized, placebo-controlled, double-blinded, community-based study in healthy children who were prescribed antibiotics for outpatient upper respiratory illnesses (URI). PLAY-ON was designed as a pragmatic study, to assess real world effectiveness of a probiotic preventing AAD. The treating clinician decided on appropriate antibiotic type, duration and dose. The study protocol was approved by the Georgetown University Institutional Review Board and registered prospectively on 9–30-2017 at ClinicalTrials.gov (NCT#03181516). The study was suspended for nearly a year during the pandemic. An independent Data Safety Monitoring Board reviewed the protocol before study initiation and at predetermined time points throughout the study. The study was conducted under IND No. 13,691 FDA/CBER.

Participants, blinding, inclusion/exclusion criteria and interventions

The probiotic strain used in this study is *Bifidobacterium animalis* subsp. *lactis*, BB-12™. BB-12 is a trademark of Chr. Hansen A/S, deposited under strain designation DSM15954 in the DSMZ - German Collection of Microorganisms and Cell Cultures. Its genome sequence is publicly available in NCBI GenBank, accession number CP001853.2. Study participants were randomized into one of two groups, allocated 1:1 to consume one serving of either 4 oz (113 ml)/d BB-12-supplemented yogurt or a non-supplemented control yogurt, in a 1:1 randomized allocation. Participants of all ages consumed the same serving of product per day. The BB-12 probiotic was not added to the control yogurt. Both probiotic and control yogurts retained the live starter cultures used in yogurt manufacture. Participants received informed consent at enrollment and began the antibiotic and yogurt interventions on Day 1. Figure 1 details the participant flow throughout the study and Fig. 2 shows the timeline of all the participant events in the study, from Day 0 to Day 180.

For information on participants, blinding, inclusion/exclusion criteria and interventions see our previous studies[17,18]. In brief, participants were healthy individuals between 3 and 12 years of age and prescribed a penicillin-class antibiotic for an acute upper respiratory infection. The control and active yogurts were manufactured at Pennsylvania State University. Both yogurts were made by fermentation using starter culture YF-L702 (Chr. Hansens, Milwaukee, WI, USA), which contained *Streptococcus thermophilus* and *Lactobacillus*

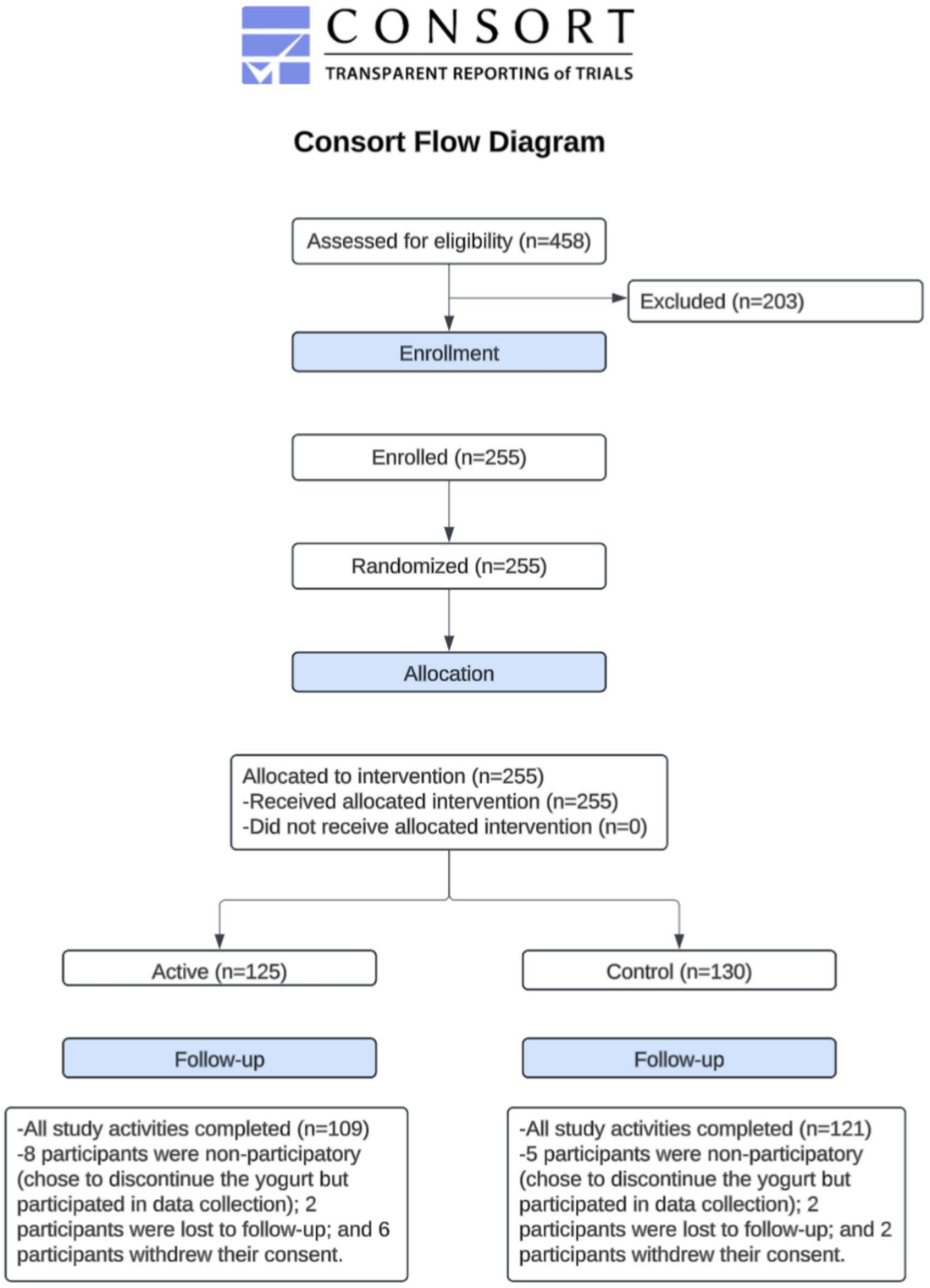

Fig. 1. Participant Flow Chart. The participant flow of the study.

bulgaricus. The active yogurt also contained $\geqslant 10^8$ cfu/g *B. lactis* BB-12, delivering a dose per serving of $\geqslant 10^{10}$ cfu/g BB-12.

Randomization

Allocation concealment of each participant was achieved using randomly allocated permuted blocks sizes of 4, 8, and 12. Research personnel had no methods to alter randomization or enrollment, nor did they have any knowledge of group assignment during the study. To check assumptions of independence and randomization,

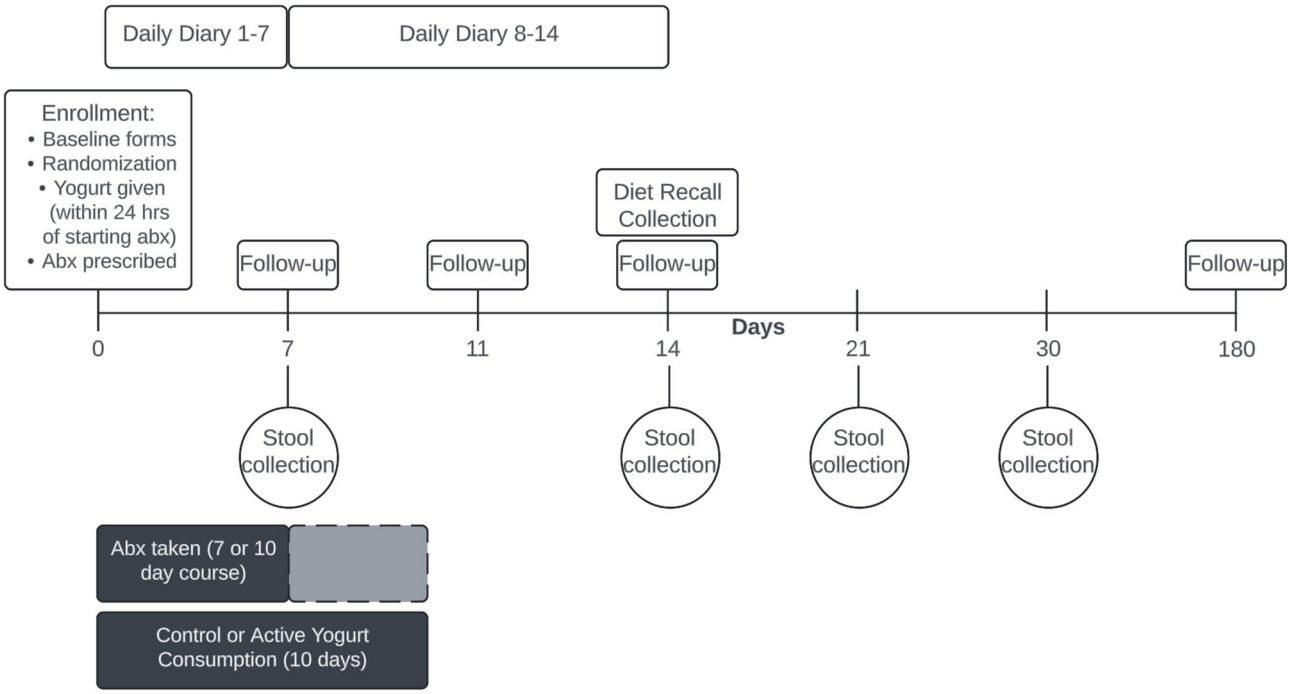

Fig. 2. Study design. The study timeline of all participant events from Day 0 to Day 180.

approximately every 6–8 months follow-up, residual analyses using the individuals who had completed the study at that time point were examined by plotting the residual by order (date) of randomization. Throughout the study, no patterns were observed in these plots.

Sample size calculation for the primary outcome

The primary outcome was the presence or absence of clinically diagnosed diarrhea. The rate of AAD in the general population was estimated to be 20–35%. A Cochrane review reported the incidence of AAD in a probiotic group to be 8% versus 22% in a control group[26]. Using the Cochrane estimates, we estimated the total sample size approximately 260 (132 per group (118 plus 1-% lost to follow up)) would still provide at least 80% power for detecting the difference in AAD rates of 0.14 (alpha = 0.05, two tailed).

Compliance

We assessed adherence to the interventions in two distinct manners. First, we reviewed the daily assessment diaries. Second, participants were asked to quantify their antibiotic and yogurt intake during follow-up phone calls we made each day of the intervention period. We defined protocol compliance as the consumption of 2 or more ounces of the assigned study yogurt per day, for at least 10 of the 14 days, and >80% of the total antibiotics prescribed (participants took 7 or 10 days of antibiotics).

Diarrhea definition

The definition for the primary outcome of diarrhea was 3 or more loose stools for 2 consecutive days. This is a conservative research definition in comparison to the definition used by the NIH, which is passing loose, watery stools three or more times a day[27,28].

Using adverse events (AEs) daily diaries and follow up form data, we applied three additional diarrhea definitions: at least 1 loose stool; two or more loose stools within 24 h; and three or more loose stools within 24 h.

The Bristol Stool Chart (Figure S1)was given to participants to characterize their stools[28]. Stools that are Type 6 or Type 7 are considered a loose stool.

Microbial DNA extraction

Genomic DNA was extracted from stool samples with the MagAttract microbial DNA kit (QIAGEN) using a custom automated protocol on the Hamilton Microlab STAR. Samples were thawed on ice and a 200 µL aliquot from the stool sample was used as input for the kit following the manufacturer's protocol. Cells were lysed by bead beating on the TissueLyser (QIAGEN) at 20 Hz for 20 min and the final elution volume was 110 µL[1[,23],.,

16 S rRNA gene sequence amplification and analysis

The V3–V4 regions of the 16 S rRNA gene were amplified by two-step PCR, with amplicon pooling, sequencing on an Illumina HiSeq 2500 instrument, and sequence data processing as previously described[29]. Sequence reads from the 16 S rRNA gene profiling are available on NCBI Sequence Read Archive under accession number PRJNA1079705.

Amplicon sequence variants (ASVs) generated by DADA2 were taxonomically classified using the RDP Naïve Bayesian Classifier[30] trained with the SILVA v128 16 S rRNA gene database[31] as implemented in the dada2 R package[32]. Negative controls generated a negligible amount of sequencing reads, whereas the positive controls generated the expected mock community[33].

Shotgun metagenomics

Shotgun metagenomic sequence libraries were constructed from the DNA extracts using Illumina Nextera XT Flex kits according to manufacturer recommendations and then sequenced on an Illumina HiSeq 4000 platform (150 bp paired-end mode) at the Genomic Resource Center at the University of Maryland School of Medicine. Each sample was uniquely barcoded in each HiSeq 4000 lane, yielding an average of 50 million read pairs for each sample.

Quality control of each metagenome was performed using tools from the BBMap software package[34]. Identical duplicates were removed using the Clumpify tool in "dedupe" mode allowing 0 substitutions. BBDuK was run once to remove reads from the PhiX spike-in and synthetic molecules (k = 31). BBDuK was run again to trim low-quality bases, remove adapters, and discard short reads (k = 23, mink = 11, hdist = 1, qtrim = rl, trimq = 10, minlength = 60). BBWrap was used to remove reads mapping to a human genome masked for broadly conserved sequences[35].

Taxonomic and functional profiling was performed using tools from the bioBakery 3 suite.6 Taxonomic profiles were estimated by mapping reads to clade-specific marker genes using Metaphlan version 3.0.13. Gene families and pathways were then profiled using HUMAnN version 3.0.0. For each metagenome, reads were mapped to the ChocoPhlAn pangenomes of the species identified during taxonomic profiling. A translated search was then performed against the full UniRef90 database for unmapped reads. The results were combined to yield abundance profiles of gene families. Pathway abundances and coverages were computed based on MetaCyc pathway definitions. Gene family abundances were depth-normalized to CPM (Counts Per Million) and relative abundances using the "humann_renorm_table" script included with HUMAnN. Shotgun metagenomics sequence reads are available on NCBI Sequence Read Archive under accession number PRJNA1079705.

Antibiotic resistance profiling

Relative abundance of antibiotic resistance genes was calculated using ShortBRED[36].Software script ShortBRED_quantify.py was used to profile metagenomes for markers of proteins of interest. We used the provided marker collections built on the Comprehensive Antibiotic Resistance Database (CARD)[37].ShortBRED measured gene abundance in reads per kilobase of genome per million bases of recruited sequences (RPKM). Abundances were grouped and summed in R, according to CARD resistance mechanism and drug class ontologies.

Statistical analyses of the microbiome data

Statistical analyses were performed using R (version 4.1.2). The R package car[38] was used to compute Levene's tests for homogeneity of variance analysis across groups, treatments, and time points. The R package stats[39] was used to perform the Shapiro-Wilk test of normality and to compute t-tests. The R package phyloseq[40] was used for analysis of the microbial community data and to calculate the Shannon diversity for each sample. Shannon diversity was compared between each time point within groups and treatments using paired t-tests, and the percent Shannon diversity was compared between groups at each time point using unpaired t-tests. Principal coordinates analysis (PCoA) using Bray-Curtis dissimilarity was performed to assess the beta diversity. Permutational multivariate analysis of variance (PERMANOVA) was conducted to test whether the bacterial communities sequenced have different centroids based on assignment treatment. To determine whether assumptions are met for PERMANOVA, a test of heterogeneity (ensure homogenous dispersion) was performed. The microbiota composition calculations were performed on filtered data that contained samples with a minimum of 3,000 reads, had singletons removed, each ASV was prevalent in at least 20% of samples, and taxa with less than 1% relative abundance were removed. Differences between groups and treatments at each time point were evaluated using metagenomeSeq computed using the R package microbiomeMarker[41] Cumulative sum-scaling normalization was applied, which attempts to normalize sequence counts based on the lower-quartile abundance of features. We then used a zero-inflated Gaussian (ZIG) mixture model for differential analysis. The *p*-values were Bonferroni adjusted for multiple comparisons with a *p*-value cutoff of 0.05. Mann-Whitney U tests were used to compare the species-level data. The R packages ggplot2[42] and ComplexHeatmap[43] were used to compute the visualizations of the relative taxa abundances. Correlation analysis was performed with the Pearson's test (two-tailed) at the significance level $p < 0.05$.

Results

Participant demographics

Study enrollment took longer than anticipated due closures imposed during the pandemic. There were no clinically significant differences between the groups (Table 1). The average ages for the active and control groups were 7.0 and 6.3 years, respectively, with 50% of participants being female. Hispanics comprised 45% of the participant population. Most (74%) participants in the study were prescribed amoxicillin. Up to 10 of the participants delayed the first dose of probiotic by ≥ 24 h.

Primary outcome: incidence of diarrhea

The incidence of diarrhea (Table 2) in our study was 2% in both groups ($p = 0.62$), which is much lower than the 20–35% that has been reported. Using the metric of loose stools, we also found no difference between active and control groups, reporting 20% and 14% ($p = 0.19$), respectively. Loose stools were stools that received a grade of

Group		Active (*N* = 125)	Control (*N* = 130)	Total (*N* = 255)
Age	Average (years)	7.0	6.4	6.7
Sex	Male	53	64	117
	Female	65	62	127
	Unknown	7	4	11
Racial background	White	77	82	159
	Black or African American	2	3	5
	Asian	4	4	8
	American Indian or Alaska Native	0	1	1
	More than one race	19	17	36
	Not reported*	17	12	29
	Unknown**	6	11	17
Ethnicity (Hispanic or Latino origin)	Yes	58	58	116
	No	61	68	129
	Not reported	1	0	1
	Unknown	5	4	9
Health insurance	Yes	110	123	233
	No	10	4	14
	Unknown	5	3	8
Smoking in the household	Yes	7	2	9
	No	112	125	237
	Unknown	6	3	9
Marital status of accompanying guardian	Single	16	12	28
	Married	82	92	174
	Living with a partner	13	6	19
	Divorced	4	2	6
	Separated	2	5	7
	Widowed	0	1	1
	Not reported	3	9	12
	Unknown	5	3	8
Annual income	< $15,000	7	6	13
	$15,000 - $30,000	18	8	26
	$30,001 - $50,000	10	8	18
	$50,001 - $75,000	5	7	12
	$75,001 - $100,000	4	5	9
	$100,001 - $150,000	17	15	32
	$150,001 - $200,000	10	15	25
	> $200,000	29	29	58
	Prefer not to answer	20	34	54
	Unknown	5	3	8
Antibiotic prescribed	Amoxicillin	88	100	188
	Amoxicillin-clavulanate	9	6	15
	Cefdinir	11	15	26
	Penicillin	4	3	7
	Cephalexin	3	1	4
	Unknown	10	5	15

Table 1. Participant demographics. *Not reported refers to participants who chose not to report their answer. **Unknown refers to participants who did not answer the question.

6 or 7 on the Bristol Stool Chart[28]. There was no significant difference (BB-12 compared to control) in diarrhea defined using the additional post hoc definitions (Table 3)**.**

The incidence of diarrhea in individuals who received amoxicillin or penicillin (74% of total participants) was 0.5%. However, the incidence of diarrhea in individuals who received more broad spectrum antibiotics, which included amoxicillin-clavulanate, cefdinir, or cephalexin, was 9.1% (p-value of 0.004 and Fisher's exact test probability of 0.0044 for the hypothesis that the rate of diarrhea is different across the two groups of antibiotics).

Group	Active (*N* = 125)	Control (*N* = 130)	Total (*N* = 255)
Diarrhea*	3 (2%)	2 (2%)	5 (2%)
Loose Stools**	25 (20%)	19 (14%)	44 (17%)

Table 2. Reported episodes of diarrhea (primary outcome from AE forms) and loose stools (post hoc outcome from information provided on AEs forms). *Defined as 3 or more loose stools for 2 consecutive days. **Defined as stools graded a 6 or 7 on the Bristol Stool Scale. ***$p = 0.62$ for diarrhea and 0.19 for loose stools.

Form	No. episodes diarrhea defined as 3 or more loose stools for 2 consecutive days	No. episodes diarrhea defined as ≥ 1 loose stool	No. episodes diarrhea defined as ≥ 2 loose stools within 24 h	No. episodes diarrhea defined as ≥ 3 loose stools within 24 h
AEs	Active: 3 Control: 2	Active: 26 Control: 21		
DD 1–7 & 8–14 (*N* = 315)		Active: 39 Control: 44	Active: 9 Control: 9	Active: 1 Control: 5
FU D7 (*N* = 233)		Active: 19 Control: 24	Active: 2 Control: 13	Active: 1 Control: 3
FU D11 (*N* = 206)		Active: 12 Control: 13	Active: 2 Control: 3	Active: 1 Control: 2
FU D14 (*N* = 218)		Active: 12 Control: 12	Active: 6 Control: 2	Active: 3 Control: 2

Table 3. Reported episodes of diarrhea using post hoc diarrhea definitions. Time of stool is not noted on follow-up forms, so it could not be checked if the stools are within exactly 24 h. The stools noted within 24 h are either on the same day or consecutive days. The times were checked for all daily diary stools. AE, adverse events; DD, daily diary; FU, follow-up.

Group	Active (*N* = 125)	Control (*N* = 130)	Total (*N* = 255)
Other	28	43	71(20%)
Loose Stools	25	19	44 (17%)
Constipation	15	21	36 (10%)
Cough	13	17	30 (8%)
Nasal Congestion	14	12	26 (7%)
Fever	11	14	25
Pain	11	9	20
Runny Nose	10	9	19
Lack of Appetite	8	10	18
Sore Throat	5	13	18
Rash	4	10	14
Flatulence	6	4	10
Vomiting	8	2	10
Earache	6	1	7
Diarrhea	3	2	5
Total Number of AEs	167	185	352
Total Number of People with AEs	64	69	133

Table 4. Total number and type of adverse events in decreasing order of frequency. Event rate of total AEs is 1.3 in active versus 1.4 in control, ($p = 0.56$). *Total number of people with AEs was 0.51 vs. 0.53 (p-0.76) in the active vs. control groups.

Secondary outcome: safety

Active and control participants reported 167 and 185 AEs, respectively. The most common AEs were loose stools (17% of all AEs events), constipation (10%), cough (8%) and nasal congestion (7%). It is important to remember all participants were sick with an underlying acute respiratory infection. No significant difference in AEs was observed between groups (Table 4).

Group	Active ($N=125$)	Control ($N=130$)	Total ($N=255$)
Number of participants who took ≥ 80% of abx	78 (62.4%)	91 (70.0%)	169 (66.3%)
Number of participants who took ≥ 10 days of yogurt	61 (48.8%)	71 (54.6%)	132 (51.8%)
Number of participants who took ≥ 7 days of yogurt	86 (68.8%)	93 (71.5%)	179 (70.2%)

Table 5. Participant compliance with antibiotic and yogurt intake.Total number of participants who took > 80%, $p=0.20$; number of participants who took > 10 days, $p=0.35$; number of participants who took > 7 days of yogurt, $p=0.44$.

Group	Study Day				
	0	7	14	21	30
BB-12	84	85	80	76	69
Control	96	88	90	81	79

Table 6. Sample distribution between the two treatments (BB-12 and control) and five time points that were processed for 16 S rRNA gene sequencing.

	Day 0 → Day 7		Day 0 → Day 14		Day 0 → Day 21		Day 0 → Day 30		Day 7 → Day 14	
	t-test	*p*-value	t-test	*p*-value	t-test	*p*-value	t-test	*p*-value	t-test	*p*-value
BB-12	3.981	< 0.001	−0.562	0.576	−2.145	0.035	−3.337	0.001	−6.465	< 0.001
Control	5.427	< 0.001	0.631	0.530	−0.013	0.990	0.179	0.859	−4.199	< 0.001
	Day 7 → Day 21		Day 7 → Day 30		Day 14 → Day 21		Day 14 → Day 30		Day 21 → Day 30	
	t-test	*p*-value	*t*-test	*p*-value	*t*-test	*p*-value	*t*-test	*p*-value	*t*-test	*p*-value
BB-12	−7.506	< 0.001	−7.952	< 0.001	−2.219	0.030	−2.451	0.017	−0.025	0.980
Control	−6.700	< 0.001	−5.530	< 0.001	−1.708	0.092	−0.936	0.353	0.542	0.590
	Day 0		Day 7		Day 14		Day 21		Day 30	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BB-12	3.835	0.545	3.567	0.536	3.862	0.474	3.935	0.394	3.975	0.419
Control	3.925	0.538	3.612	0.483	3.86	0.476	3.974	0.435	3.901	0.456

Table 7. t-test results describing percent alpha diversity changes (Shannon index) between the control and BB-12 treatments using the 16 S rRNA gene sequencing data. Percent change within each treatment was calculated comparing the Shannon diversity index at each follow-up day (i.e., day 7, day 14, day 21, and day 30) to day 0. Mean and standard deviation (SD) shown for day 7, day 14, day 21, and day 30 for each probiotic/control treatment.

Compliance

We measured compliance by tracking both antibiotic and yogurt intake. In the active group, 62% of participants reported taking over 80% of their antibiotic doses compared to 70% in the control group. Yogurt compliance, defined as taking yogurt for ≥ 7 days, was nearly identical for each group at 68.8% for active and 71.5% for control (Table 5).

16 S amplicon sequencing analysis

16 S rRNA gene sequence output. Gut microbiota analyses were carried out on fecal samples. In total, 11,881 ASVs and 31,617,089 reads were identified in 850 analyzed fecal samples (average of 37,197 reads per sample). Twenty-two samples that exhibited fewer than 3,000 reads were removed, resulting in a total of 828 fecal samples available for downstream analysis (Table 6).

BB-12 recipients present with richer diversity post-treatment. We compared the Shannon diversity in fecal samples obtained on day 0 and day 7 within each treatment group and observed a significant decrease in diversity for both treatments (BB-12 and control; *t*-test, both $p < 0.001$; Table 7; Fig. 3A). The Shannon diversity recovers to pre-treatment levels by day 14 in both treatment arms of the study (Table 7; Fig. 3A). When we compared the Shannon diversity in samples from BB-12 recipient versus control recipients over time, the percent diversity changes at each time point compared to baseline were not statistically significant (*t*-test, all $p > 0.05$; Table S1, Fig. 3B). Nonetheless, the decrease in Shannon diversity observed at day 7 is approximately 1.3-fold larger in the control treatment (−7.174%, Table S1) compared to the BB-12 treatment group (−5.604%, Table S1). Moreover,

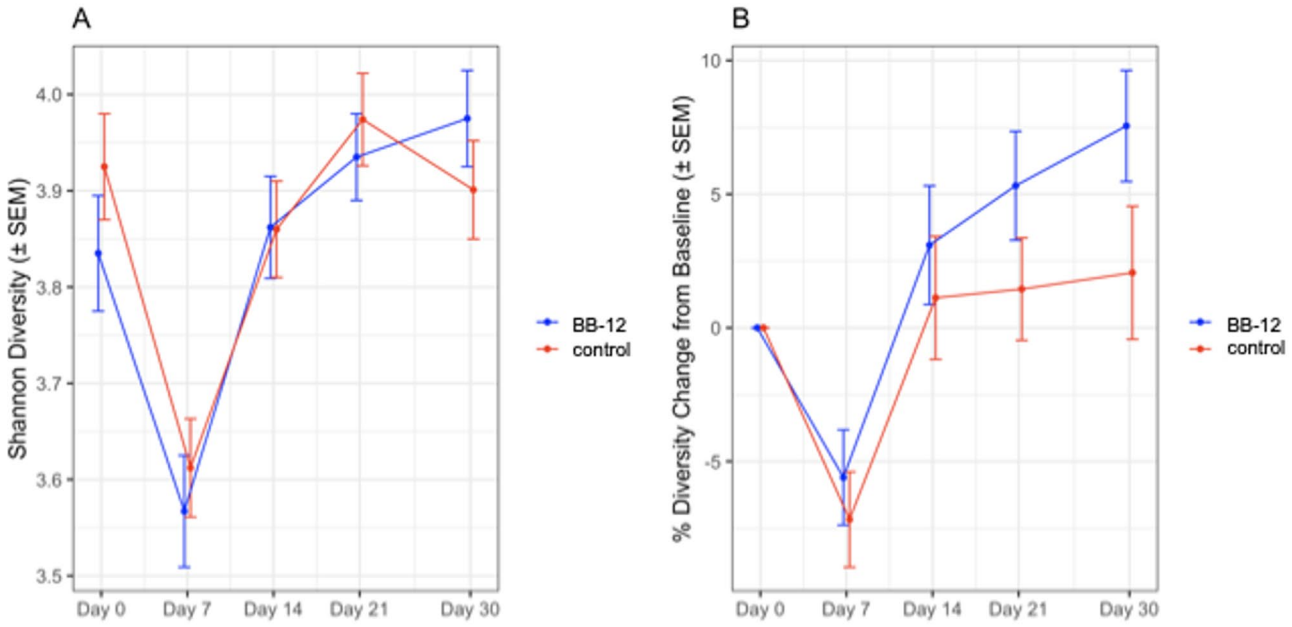

Fig. 3. Longitudinal alpha diversity changes (± SEM) for each treatment using the 16 S rRNA gene sequencing data. **(A)** Shannon diversity change from day 0 through day 30 for BB-12 recipients and the control group. **(B)** Percent Shannon diversity change compared to day 0 for each of the two treatments BB-12 and control.

	Study Day				
Group	**0**	**7**	**14**	**21**	**30**
BB-12	46	46	44	45	46
Control	50	49	50	48	50

Table 8. Breakdown of the subset of participants and samples included in metagenomic analyses. This subset comprises participants with baseline and all follow-up samples, after bioinformatic quality-control filtering.

the BB-12 recipients exhibit an increase in diversity longitudinally compared to the control recipients, which results in a 3.7-fold larger increase in percent diversity change at day 30 (Fig. 3B, Table S1).

Shotgun metagenomics analysis

Alpha diversity trends between 16 S amplicon sequencing and shotgun metagenomics are similar. To further investigate the impact of antibiotics, with or without BB-12 administration, on the gut microbiome, we performed metagenomic sequencing on participants with complete longitudinal sample sets (baseline/day 0 and all follow-up timepoints: days 7, 14, 21, and 30). This included 46 participants in the BB-12 group and 50 participants in the control group. The number of samples retained at each timepoint after bioinformatic quality-control processing is slightly lower than the number of participants because some samples did not pass QC (Table 8). The alpha diversity calculated using the shotgun metagenomics data from this subset of samples exhibits similar trends to the alpha diversity that was calculated using 16 S rRNA gene sequencing data from the complete cohort (Fig. 4A; Table 9). The percent diversity changes at each time point compared to baseline in the metagenomic sequencing dataset also did not show any significant differences when BB-12 recipients were compared to control recipients (*t*-test, all $p > 0.05$; Fig. 4B; Table 10). T-tests comparing the percent alpha diversity changes of the subset analyzed using metagenomic sequencing were similar when compared to the diversity changes observed using 16 S rRNA gene sequencing (16 S) (Figure S2, Table 11).

Antibiotic administration causes reduction in Firmicutes and increase in Bacteroides. Principal coordinates analysis (PCoA) using Bray-Curtis dissimilarity method and PERMANOVA suggest that both the study day and treatment contributes significantly to the variance of the community composition (Fig. 5; Table 12).

The longitudinal changes in taxonomic composition at the phylum level show that at day 7 the Firmicutes were reduced and the Proteobacteria were increased in both treatments. However, this shift was more pronounced in the control recipients compared to BB-12 (Fig. 6; Table 13).

In order to determine whether any species exhibited significant abundance changes within each of the genera represented (Fig. 7), we used metagenomeSeq. Few differences were statistically significant; however, a species of the genus *Coprobacter* was significantly enriched at days 0 and 7 in the control recipients (Fig. 8; Table 14), at day 7 *Eubacterium* sp. CAG 180 was more abundant in the BB-12 recipients, and at day 30 *Barnesiella intestinihominis* was enriched in the control group (Fig. 8; Table 14).

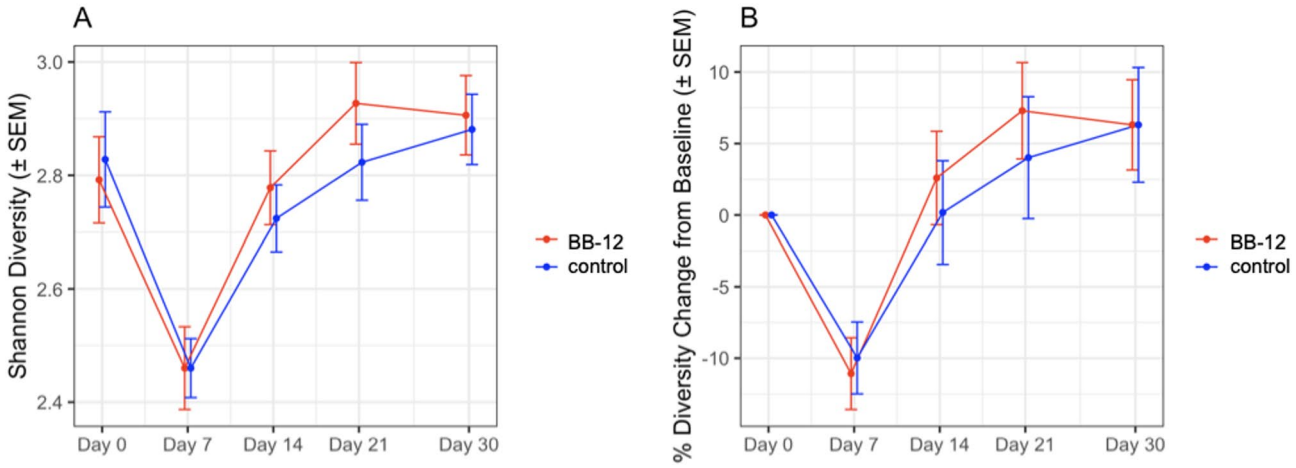

Fig. 4. Longitudinal alpha diversity changes (± SEM) for each treatment calculated using the whole genome sequencing data from the subset that underwent whole genome sequencing (Table 7). **(A)** Shannon diversity change from day 0 through day 30 for each of the treatments (BB-12, control). **(B)** Percent Shannon diversity change compared to day 0 for each of the two treatments (BB-12, placebo). **(C)** Shannon diversity change from day 0 through day 30 for the BB-12 recipients and the control group.

	Day 0 → Day 7		**Day 0 → Day 14**		**Day 0 → Day 21**		**Day 0 → Day 30**		**Day 7 → Day 14**	
	t-test	***p*-value**	**t-test**	***p*-value**	**t-test**	***p*-value**	**t-test**	***p*-value**	**t-test**	***p*-value**
BB-12	4.853	< 0.001	−0.015	0.988	−1.720	0.093	−1.679	0.100	−3.982	< 0.001
Control	5.070	< 0.001	1.229	0.225	0.107	0.915	−0.603	0.549	−4.555	< 0.001
	Day 7 → Day 21		Day 7 → Day 30		Day 14 → Day 21		Day 14 → Day 30		Day 21 → Day 30	
	t-test	*p*-value	*t*-test	*p*-value	*t*-test	*p*-value	*t*-test	*p*-value	*t*-test	*p*-value
BB-12	−5.595	< 0.001	−5.667	< 0.001	−2.271	0.028	−1.921	0.061	0.414	0.681
Control	−5.434	< 0.001	−8.719	< 0.001	−1.890	0.065	−2.525	0.015	−1.124	0.267
	Day 0		Day 7		Day 14		Day 21		Day 30	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BB-12	2.792	0.514	2.460	0.493	2.778	0.433	2.927	0.485	2.906	0.476
Control	2.828	0.595	2.460	0.365	2.724	0.42	2.823	0.468	2.881	0.438

Table 9. ***t***-test results describing alpha diversity differences (Shannon index) between the BB-12 and control recipients using metagenomic sequencing data. Mean and standard deviation (SD) shown for day 0, day 7, and day 14.

	Control vs. BB-12		**Control**		**BB-12**	
	t-test	***p*-value**	**Mean**	**SD**	**Mean**	**SD**
Day 7	0.309	0.758	−9.982	17.581	−11.079	17.011
Day 14	0.490	0.625	0.179	25.594	2.594	21.636
Day 21	0.599	0.551	4.014	29.507	7.296	22.602
Day 30	0.000	1.000	6.306	28.368	6.307	21.398

Table 10. t-test results describing percent alpha diversity changes (Shannon index) between the BB-12 and control treatments using metagenomic sequencing data. Percent change within each treatment group was calculated comparing the Shannon diversity index at each follow-up day (i.e., day 7, day 14, day 21, and day 30) to day 0. Mean and standard deviation (SD) shown for day 7, day 14, day 21, and day 30 for each BB-12/control treatment group.

	Control		BB-12	
	16 S vs. WGS		16 S vs. WGS	
	t-test	*p*-value	*t*-test	*p*-value
Day 7	0.931	0.353	1.819	0.071
Day 14	0.231	0.818	0.130	0.896
Day 21	0.617	0.539	0.535	0.594
Day 30	0.949	0.345	0.342	0.733

Table 11. ***t***-test results describing percent alpha diversity changes (Shannon index) for each treatment between the metagenomic sequencing data from the subset and the 16 S rRNA gene sequencing (16 S) data from the whole cohort. Percent change within each group was calculated comparing the Shannon diversity index at each follow-up day (i.e., day 7, day 14, day 21, and day 30) to day 0.

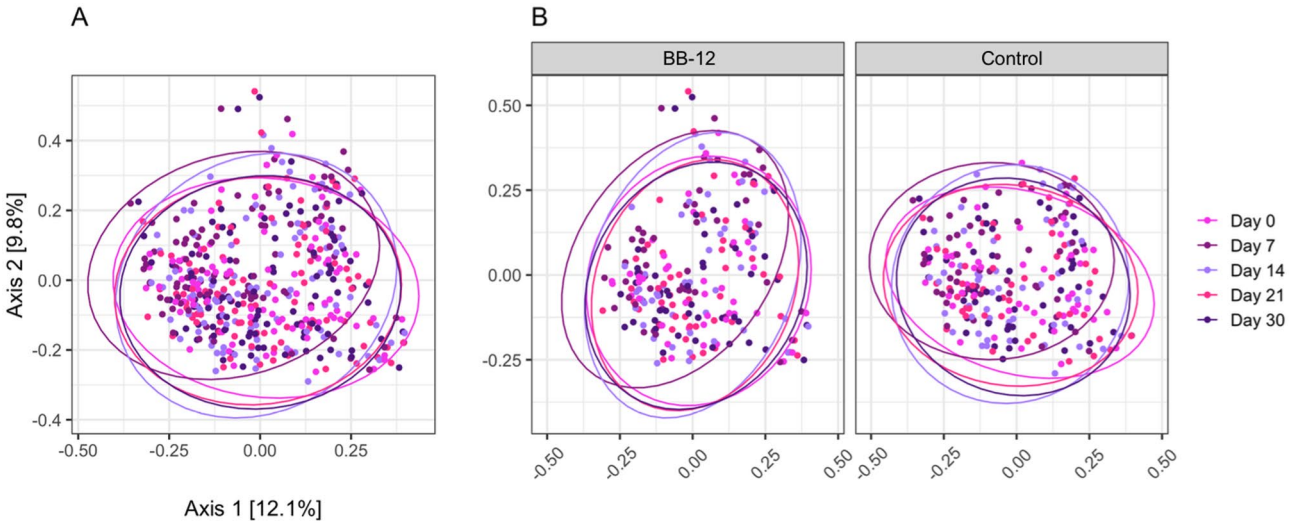

Fig. 5. Principal coordinate analysis (PCoA) using Bray-Curtis dissimilarity displaying the beta diversity changes across the 30 day study period using the WGS data. **(A)** PCoA showing the community composition shifts across the five study time points. **(B)** Individual PCoA plots displaying the longitudinal beta diversity changes of the BB-12 recipients (left) and the control group (right).

Main effects	df	SS	pseudoF	R^2	$P_{(perm)}$*
Treatment	1	0.586	2.320	0.005	**0.001**
Study Day	4	2.920	2.889	0.023	**0.001**
Interaction terms					
Treatment x Study Day	4	0.290	0.287	0.002	0.835
Residual	484	122.300		0.970	
Total	493	126.096		1.000	
*Significance values based on 999 permutations.					

Table 12. PERMANOVA results for the comparison of the different gut microbial communities across the study period between BB-12 and control recipients based on Bray-Curtis distances using metagenomic sequencing data. SS, sum of squares.

Bifidobacterium animalis was more abundant in BB-12 recipients compared to control. We classified *Bifidobacterium* to the species level to determine whether BB-12 could be detected using metagenomic sequencing data. The relative abundance of *Bifidobacterium animalis* increased significantly in the BB-12 compared to control treatments at days 7 and 14 (Fig. 9 and Table 15; Mann-Whitney U, $p < 0.001$ and $p = 0.016$, respectively).

Significant shifts in antibiotic resistance genes during antibiotic administration. To assess and quantify the repertoire of ARGs in the gut microbiome of the study participants, we conducted a ShortBRED analysis[36] using CARD[37]. In total, we identified 259 ARGs, which conferred resistance to 34 drug classes categorized into seven

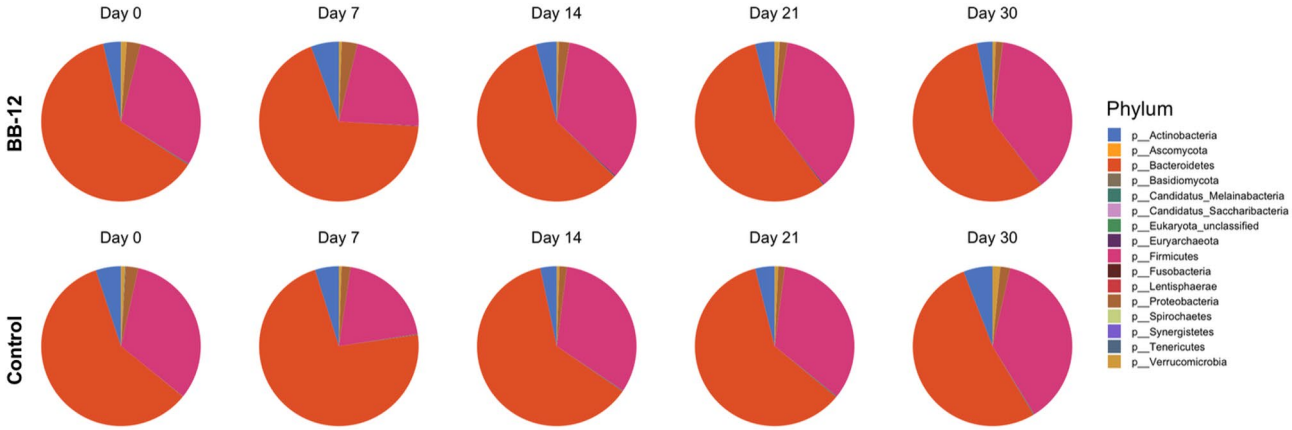

Fig. 6. Pie charts displaying the relative longitudinal taxonomic changes at the phyla level using the WGS data. Top row, BB-12 recipients; bottom row, control group.

	Study Day 0		Study Day 7		Study Day 14		Study Day 21		Study Day 30	
Phylum	BB-12	Control	BB-12	Control	BB-12	Control	BB-12	Control	BB-12	Control
p__Actinobacteria	3.6233	5.1135	5.6908	4.8399	4.2348	3.3304	3.9626	3.9419	3.1672	5.9133
p__Ascomycota	0.0001	0.0000	0.0000	0.0001	0.0004	0.0002	0.0000	0.0000	0.0000	0.0000
p__Bacteroidetes	62.3131	59.0291	68.3598	72.4633	58.6672	62.1246	56.4508	60.1065	57.2826	52.7006
p__Basidiomycota	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
p__Candidatus_Melainabacteria	0.1597	0.0339	0.0137	0.1192	0.0826	0.1220	0.0495	0.1647	0.0232	0.1461
p__Candidatus_Saccharibacteria	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0006
p__Eukaryota_unclassified	0.0001	0.0036	0.0001	0.0000	0.0003	0.0000	0.0000	0.0000	0.0001	0.0000
p__Euryarchaeota	0.0341	0.0079	0.0927	0.0056	0.1968	0.0108	0.1819	0.0038	0.0952	0.0138
p__Firmicutes	29.8757	32.2967	22.0846	20.1965	34.2045	32.3451	36.7053	33.6989	37.2914	37.6678
p__Fusobacteria	0.0006	0.0022	0.0085	0.0001	0.0089	0.0001	0.0039	0.0020	0.0234	0.0010
p__Lentisphaerae	0.0065	0.0107	0.0031	0.0028	0.0952	0.0050	0.0249	0.0184	0.0354	0.0069
p__Proteobacteria	2.8256	2.5079	3.1706	1.8302	2.0942	1.5179	1.6341	1.3365	1.4316	1.9760
p__Spirochaetes	0.0037	0.0000	0.0000	0.0000	0.0051	0.0000	0.0683	0.0000	0.0038	0.0000
p__Synergistetes	0.0001	0.0033	0.0000	0.0014	0.0000	0.0000	0.0000	0.0001	0.0000	0.0029
p__Tenericutes	0.0000	0.1302	0.0000	0.0000	0.0000	0.0000	0.0000	0.0125	0.0000	0.0129
p__Verrucomicrobia	1.1576	0.8609	0.5761	0.5409	0.4102	0.5439	0.9186	0.7148	0.6461	1.5582

Table 13. Longitudinal distribution of the relative abundances of the represented phyla across the five study time points using metagenomic sequencing data.

resistance mechanisms, as designated by CARD (Fig. 10, Figure S3). To determine a normalized abundance for each ARG we calculated the RPKM.

Total ARG RPKMs. Since the changes in ARG abundances were quantified and normalized using the RPKM, we compared the total ARG RPKMs for each treatment (Fig. 11) across the five study time points (Fig. 12). The average ARG abundances per participant were elevated at day 0 and day 7 compared to days 14, 21, and 30. The time frame during which increased ARG levels were observed falls within the window of antibiotic administration. No significant differences were observed between the treatments at each study time point (all $p > 0.05$ Table S2) . However, the control recipients exhibited a significant reduction of total ARGs from day 7 to day 21 (t-test, $p = 0.026$; Table S3).

Discussion

We conducted a study of 255 pediatric patients who were diagnosed with a URI and prescribed an antibiotic by their treating clinician. We hypothesized that a high dose of BB-12-containing yogurt would reduce the incidence of AAD in study participants. We found no differences in clinical rates of AAD, AEs, and few differences in fecal microbiota measures between control and BB-12 treated subjects.

We based our sample size calculation on a Cochrane review that found probiotics reduced AAD from 22% in control groups to 8% in probiotic groups. Correspondingly, a fact sheet developed for health professionals by the NIH Office of Dietary Supplements advised, "*Antibiotics can disrupt the intestinal microbiome, and people who are taking antibiotics are at risk of antibiotic-associated diarrhea. Some systematic reviews and meta-analyses*

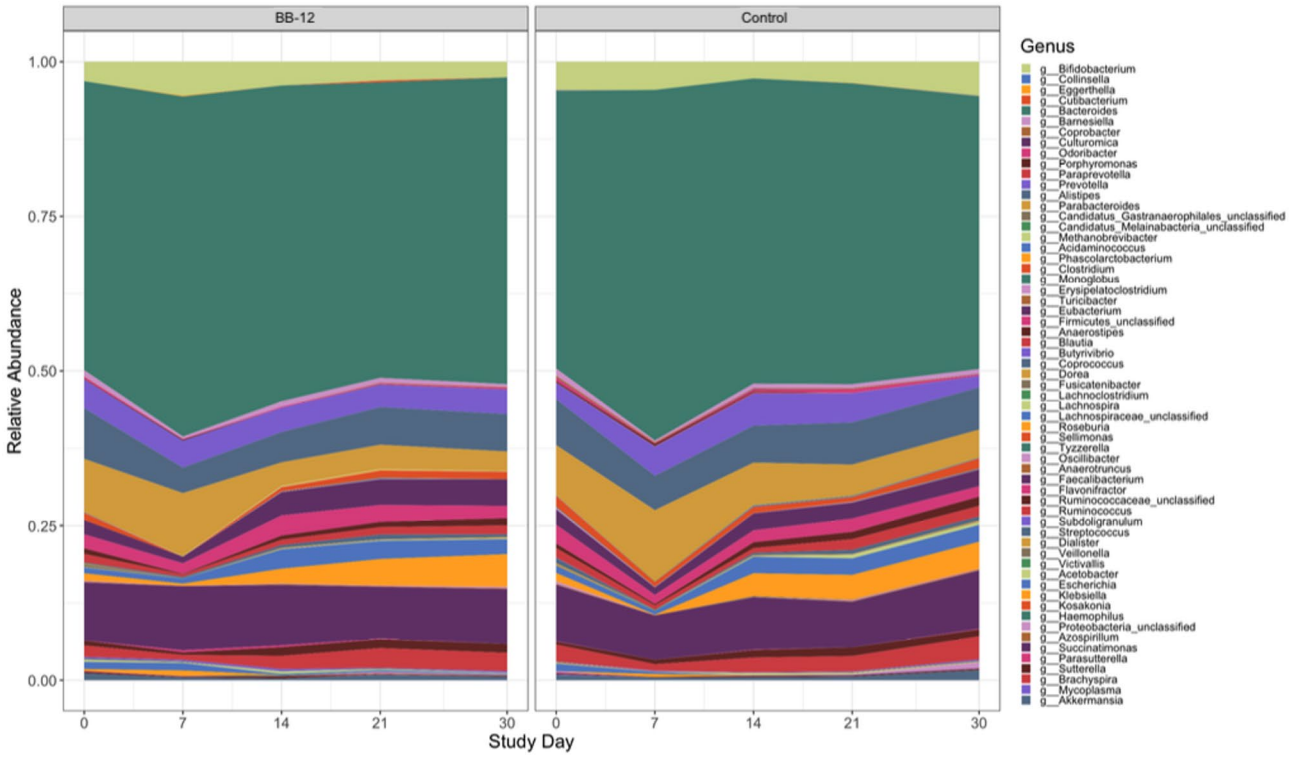

Fig. 7. Longitudinal taxonomic differences observed in the BB-12 recipients (left) and the control group (right) from day 0 to the end of the study at day 30 using the WGS data. Only genera with relative abundance > 1% are shown.

	Species	Enriched Group	log2 Fold Change	*P*-value	Adjusted *P*-value	-$\log_{10}$(*P*-value)
Day 0	**Coprobacter_sp**	**Control**	**0.376**	**4.29E-04**	**4.63E-02**	**1.335**
Day 7	Coprobacter_sp	Control	0.503	7.40E-08	8.00E-06	5.097
	Eubacterium_sp_CAG_180	BB-12	−0.612	2.36E-06	2.55E-04	3.593
Day 30	Barnesiella_intestinihominis	Control	0.866	2.48E-05	2.68E-03	2.572

Table 14. MetagenomeSeq results reporting the individual test statistic for each significant taxon/species at each timepoint for the two treatments investigated using metagenomic sequencing data (blue = BB-12; red = control). Bonferroni correction was performed for adjustment of *p*-values for multiple tests.

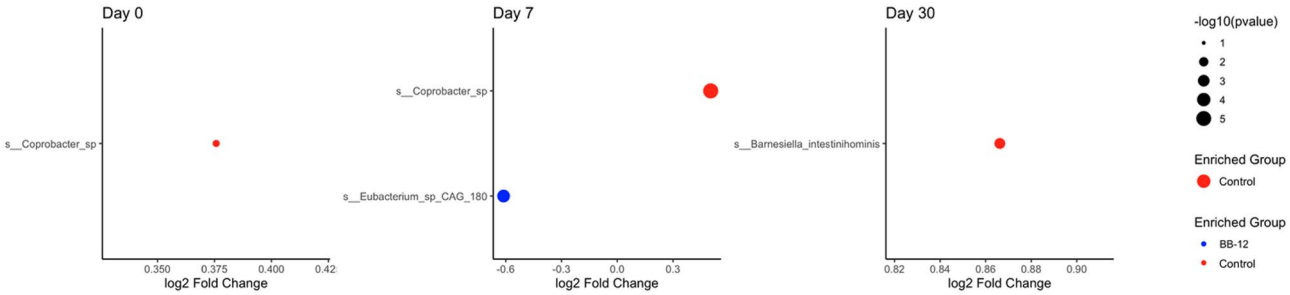

Fig. 8. Dot plots displaying significant taxa differences between the two treatments at the genus level calculated using metagenomeSeq (Bonferroni-corrected *p*-value < 0.05) using the WGS data. Each timepoint was investigated individually for the two treatments (blue = BB-12 and red = control). Dot sizes represent the -log10 of the adjusted *p*-value.

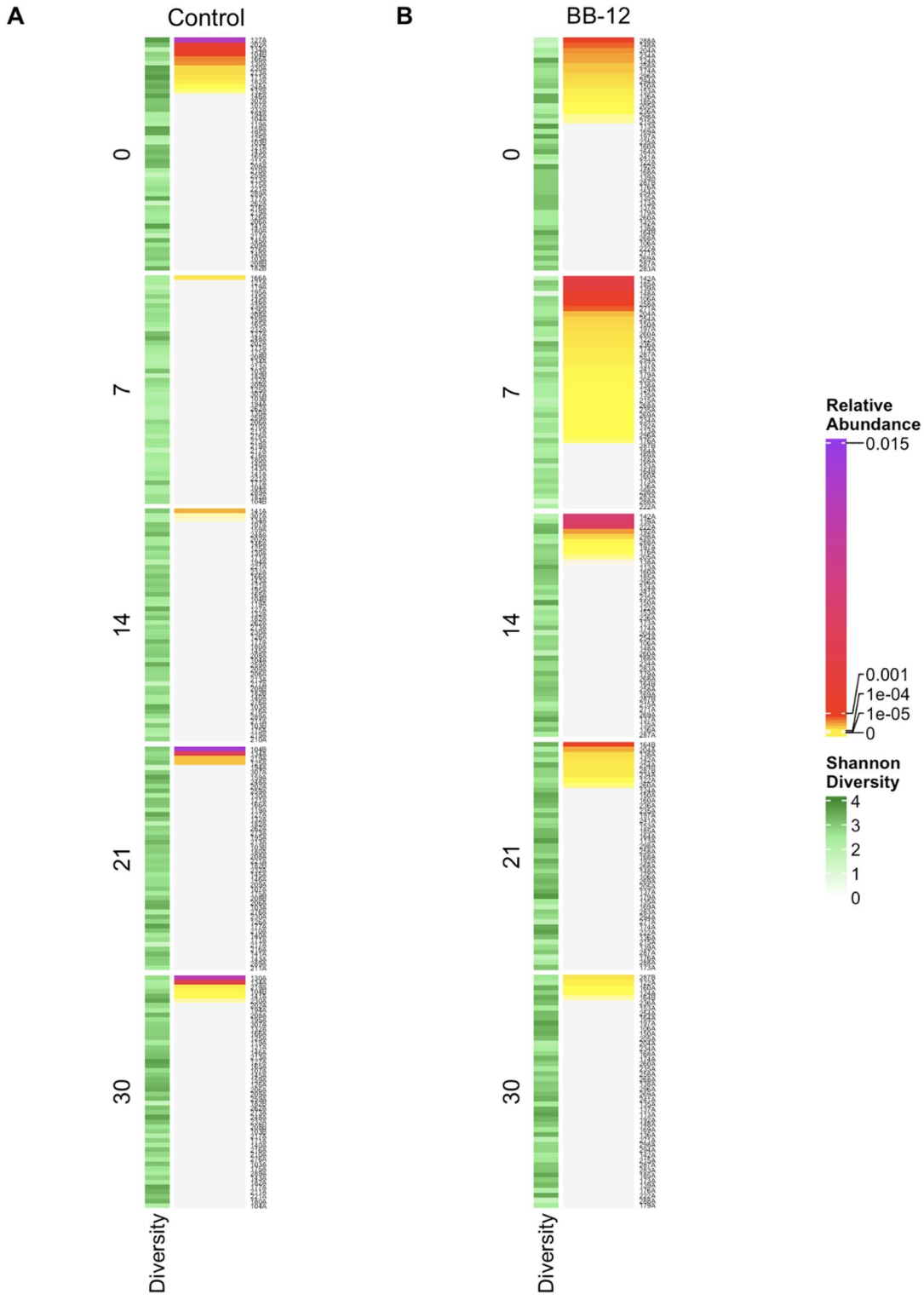

Fig. 9. Heatmaps displaying the relative abundance of *Bifidobacterium animalis* and Shannon diversity index in the **(A)** control and **(B)** BB-12 recipients. BB-12 is a member of the *Bifidobacterium animalis* species. Each study day was plotted separately with each sample shown along the y-axis. The R packages ggplot242 and ComplexHeatmap43 were used to compute the visualizations of the relative taxa abundances. ComplexHeatmap version 2.13.1 was used.

	BB-12 vs. Control	
	W-statistic	*p*-value
Day 0	1269	0.285
Day 7	1910	**<0.001**
Day 14	1291	**0.016**
Day 21	1192	0.155
Day 30	1132	0.817

Table 15. Mann-Whitney U test results describing the *Bifidobacterium animalis* relative abundance differences between BB-12 and control recipients.

report that starting certain probiotic treatments within 2 days of the first antibiotic dose helps reduce the risk of antibiotic-associated diarrhea in specific patient populations."[45] Yet, our study did not show a benefit to probiotics in preventing AAD. How do we explain our findings?

These results differ from our previous study of healthy adults, which found that probiotic yogurt mitigated disruption of the microbiota by amoxicillin-clavulante[17].The inconsistency between these findings may be due to differences in the antibiotics prescribed. A key factor that may have contributed to our null results observed is the very low (2%) rate of diarrhea we observed, which was not consistent with generally reported AAD rates of 20–35%.[7–15] A higher rate of AAD would have provided more opportunity for any potential probiotic effect to be measured. This low rate of diarrhea may have resulted from the lack of microbiome disruption we observed, which may be characteristic of the antibiotic (amoxicillin) largely prescribed by participating clinicians to subjects in our study. Our study was designed as a pragmatic clinical trial, thus community physicians determined antibiotics and dosages used. Unfortunately, this resulted in our baseline diarrhea rate being much less than we anticipated.

Our study found that amoxicillin-clavulanate reduced the Shannon diversity of the gut microbiota for all the study participants at day 7. The magnitude of this change was larger and more sustained in the control group compared to the probiotic group, which is consistent with the hypothesis that BB-12 enhanced microbiota recovery. In this current study, we did not measure fecal acetate but examined Shannon diversity and found no difference between treatment groups.

The most striking difference between our previous studies and this study is which antibiotics were used. In our previous studies, 100% of the subjects were given amoxicillin/clavulanate. In this current study, 74% of the subjects received amoxicillin and only 6% received amoxicillin/clavulanate. This difference in antibiotics could explain much of the current study's findings, and is consistent with the fact that penicillin and amoxicillin may have less impact on the microbiota and may be associated with low AAD rates[46,47].

Amoxicillin is a semisynthetic derivative of penicillin that has been used since the 1970s[48]. It irreversibly binds to and inactivates penicillin-binding protein, which is an enzyme necessary for bacterial cell wall synthesis[48]. This inactivation prevents peptidoglycan synthesis, which is critical for bacterial cell wall synthesis, leading to cell death[48]..

Amoxicillin can be used in combination with clavulanic acid, a beta lactamase inhibitor. Clavulanic acid is more protein bound and less heat stable than amoxicillin with mainly hepatic metabolism[48].With amoxicillin-clavulanate, the beta-lactam ring of clavulanic acid binds irreversibly to bacterial beta-lactamase making it more broad spectrum but also more frequently associated with gastrointestinal side effects, such as mild diarrhea and more severe *Clostridioides difficile*infection[48].

We observed very few differences in any of our microbiome measures between control and BB-12 recipients, including alpha diversity, beta diversity, taxonomic composition and ARGs. In this study and our previous study[17], we reported significant Shannon diversity differences at day 7 of antibiotic consumption, but in this study the gut microbiota in both control and probiotic treatment groups returned to baseline by day 14 and remained there through our final analysis at day 30. Additionally, our previous research demonstrated that administration of amoxicillin/clavulanate in the absence of BB-12 was associated with a significant decrease in the relative abundance of 48 taxa and BB-12 mitigated many of the taxonomic changes in the gut microbiota. These findings suggest that changes in the gut microbiota observed with a short-course of amoxicillin are minimal, precluding the usefulness for a probiotic intervention.

These conclusions are supported by the observed significant difference ($p = 0.004$) in the rates of diarrhea in participants taking more narrow spectrum ($N = 195$) versus broad spectrum antibiotics ($N = 15$). The participants who used broad spectrum penicillins had a significantly higher rate of diarrhea compared to narrow spectrum antibiotic users, 9.1% and 0.51% respectively.

The duration of antibiotic administration can also impact the structure of the microbiota and clinical outcomes. An earlier randomized, controlled trial looking at healthy volunteers found that a microbiome shift in subjects prescribed amoxicillin was most notable after 14 days of antibiotic dosing, while the maximum length of time amoxicillin was prescribed in our study was 10 days[49]. Our data support the clinical benefit to reducing prescribing days, as advocated by many public health agencies[50].

It has also been shown in mice that the gut microbiota recovers faster with shorter antibiotic treatment durations[49]. Generally, amoxicillin impacts bacterial richness and evenness, followed by a fairly rapid return of alpha diversity and abundance of major phyla after treatment. In a systematic review looking at commonly prescribed antibiotics in the UK, ten of the studies looked at amoxicillin[51]. Overall, while they observed gut microbiota changes, participants' microbiota generally recovered rapidly with five studies showing a recovery

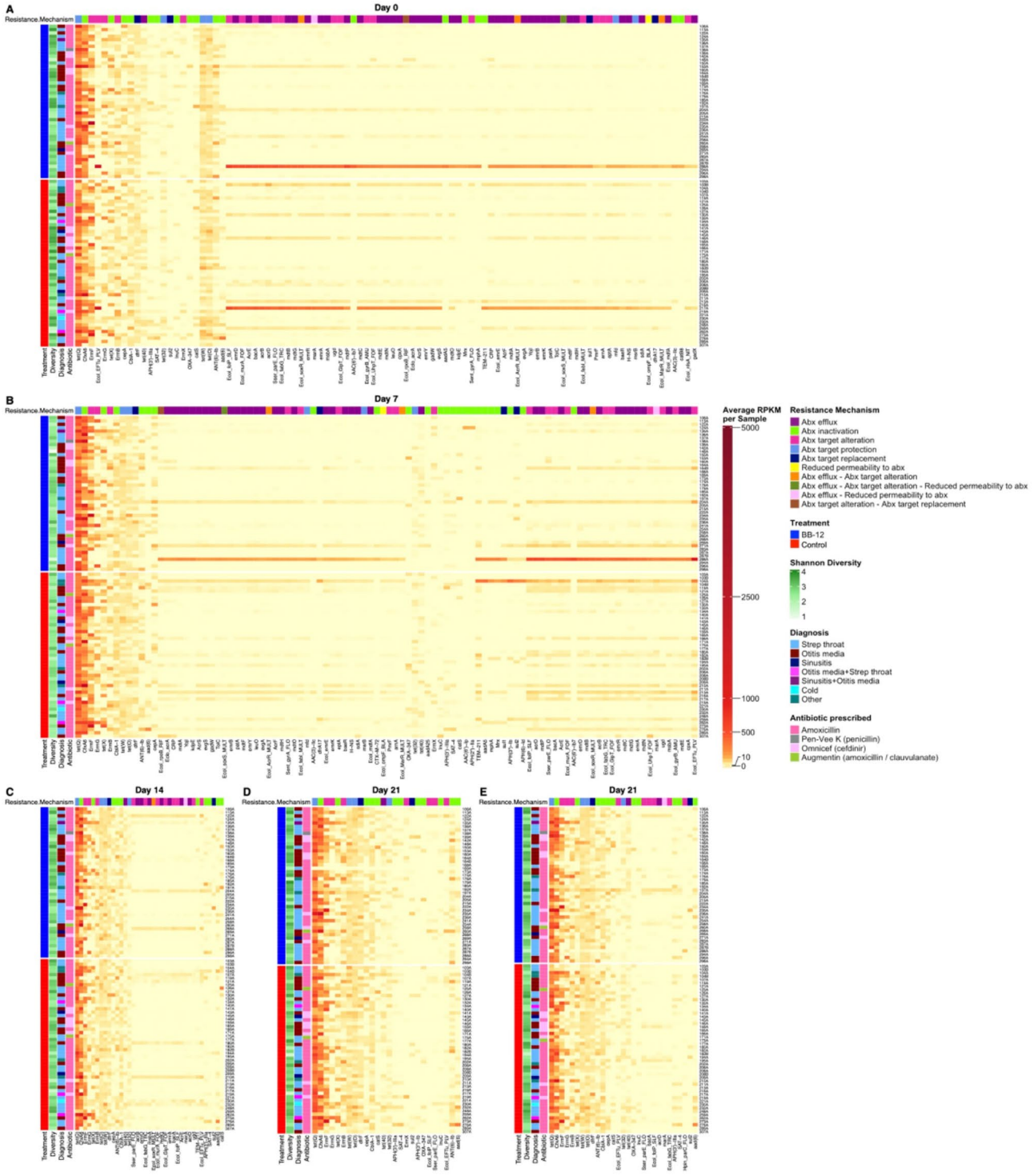

Fig. 10. Heatmaps displaying the RPKM for each ARG, treatment assignment (BB-12 or control), Shannon diversity index, diagnosis upon enrollment, and prescribed antibiotic. Each study day was plotted separately **(A)**-**(E)** with each sample shown along the y-axis. The diversity measures displayed were calculated using WGS data. Only ARGs with a total RPKM of > 100 are shown. The R packages ggplot242 and ComplexHeatmap43 were used to compute the visualizations of the relative taxa abundances. ComplexHeatmap version 2.13.1 was used.

to baseline in 2–3 weeks, and an additional study showing 6–8 weeks recovery[51]. In contrast, in subjects taking amoxicillin/clavulanate for only 5 to 7 days, the microbiota did not return to baseline for 2–8 weeks after treatment[51]. Another study in infants found that amoxicillin/clavulanate consumption caused a remodeling of bacterial microbiota with an increase in specific bacteria belonging to the Enterobacteriaceae family[52].

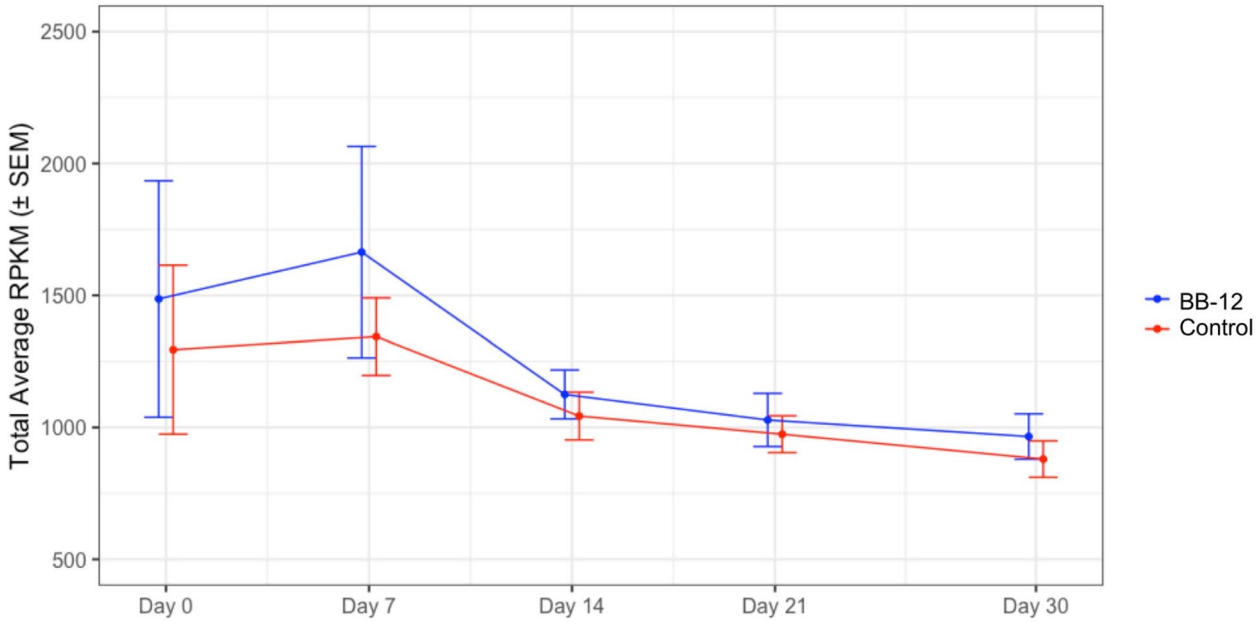

Fig. 11. Longitudinal total ARG changes (± SEM) for each treatment (BB-12, control).

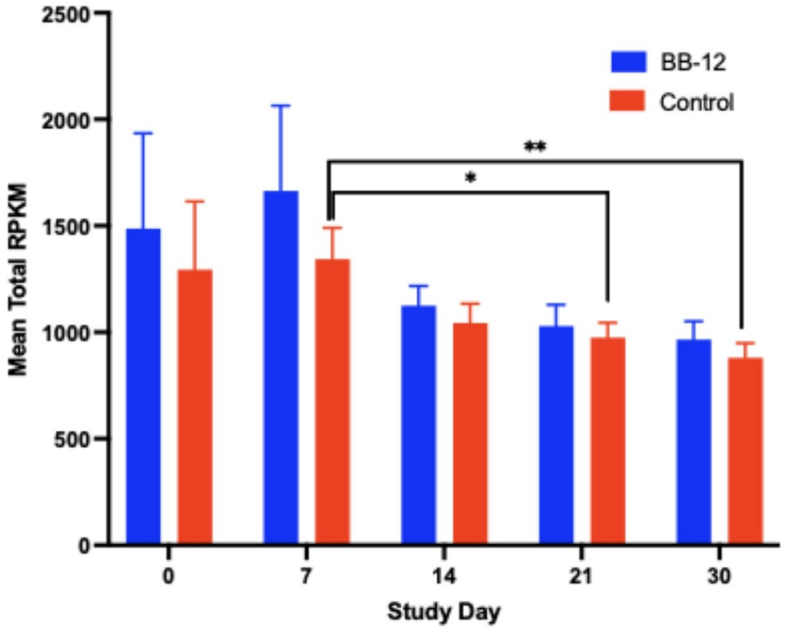

Fig. 12. Bar graphs displaying the mean total ARG changes (± SEM) for each treatment across the study period. Statistical analysis results are described in Supplementary Materials Tables S12 and S13.

Our previous studies using this same probiotic intervention in a population of adults all given amoxicillin-clavulanate found that antibiotic treatment suppressed fecal acetate levels in both the control and probiotic groups[17]. In subjects receiving probiotics, fecal acetate levels increased following cessation of antibiotic administration and returned to baseline levels on day 30 (− 1.6% baseline), whereas, in the control group, fecal acetate levels remained suppressed. BB-12 has been shown to be an acetate producer both in vitro and in vivo[26]. It has been postulated that acetate production in the gut during antibiotic administration preserves the function of key butyrate producing species via cross-feeding[53], providing a mechanism for influencing gut microbiome function and reducing AAD. However, if amoxicillin only minimally perturbs the ability of the microbiota to produce short chain fatty acids, then the potential impact of BB-12 on microbiota function may not be apparent.

Considering the substantial disparity between the effects of amoxicillin and amoxicillin/clavulanate on the microbiota's taxonomic profile, a significant impact of BB-12 on the fecal microbiota is unlikely. (Figure S4).

A previous study by Montassier et al. demonstrated in a study supplementing human subjects with antibiotics, with or without probiotics, that the probiotic increased the ARG reservoir in the human gut microbiota[54–56]. Notably, the antibiotics used in Montassier et al. were metronidazole and ciprofloxacin, which are broad spectrum drugs that cause widespread microbiota disturbances. In contrast, our results showed no increase in ARGs in either probiotic or control groups, ARG levels in our probiotic group followed the same downward trajectory that we observed in the control group, and paired analyses did not identify any increases, perhaps reflecting the lack of widespread microbiota disruption caused by amoxicillin. These findings emphasize the importance of selecting antibiotics with minimal microbiota impact whenever possible and targeting probiotic use to circumstances where benefits are most likely.

An important message from our study is the reinforcement of the long-known, but often ignored, reality in clinical practice that the type of antibiotic prescribed can have important clinical and microbiological implications. It is clear from our analysis that amoxicillin should be used as much as possible over amoxicillin/clavulanate, the latter being more disruptive to the gut microbiota. Importantly, there has been a push to limit the number of days all antibiotics are prescribed, with five days[50] being shown to be sufficient to treat many infections. Our community study showed no impact of BB-12 or antibiotics on AAD or microbiota measures, supporting the conclusion that choosing therapeutic antibiotics properly can mitigate the concerns about antibiotic-induced microbiome disruption and clinical side effects.

Limitations

The limited incidence of URIs was greatly impacted by the shutdowns and social isolation imposed during the pandemic, resulting in delays in subject recruitment. Further, we did not prescribe the antibiotics and thus some children started the probiotics up to 24 h after taking their first antibiotic dose. Reducing the time between starting the probiotic and taking the first antibiotic would be prudent. Future studies looking to assess impact on the gut microbiota should include more broad spectrum antibiotics. As in our study many different antibiotic types, doses and time frames were prescribed, and the majority of antibiotics used had minimal impact on the microbiome and do not often cause diarrhea. This resulted in the most significant limitation of our study, which was very low AAD rates.

Conclusions

Probiotics are often recommended to prevent AAD, but our study demonstrates that in the scenario where antibiotic causes little microbiota disturbance, probiotics may not provide benefit[57]. Many meta-analyses have highlighted the role of probiotics in alleviating AAD[26,58,60,60]; but subgroup analysis based on antibiotic class or spectrum of activity has not been studied to our knowledge. Such analyses could provide improved approaches to recommending interventions to alleviate AAD. Our study found no difference in incidence of AAD between the probiotic and the control yogurt.

Data availability

Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval. Please contact Ms. Tina Tan to request data. Sequence reads from the 16 S rRNA gene profiling are available on NCBI Sequence Read Archive under accession number PRJNA1079705. Shotgun metagenomics sequence reads are available on NCBI Sequence Read Archive under accession number PRJNA1079705.

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Author contributions
All authors contributed to writing the main manuscript text and all authors reviewed the manuscript. C.F. and S.G. led the microbiome analysis. C.F. and S.G. prepared Tables 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15; Figs. 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. D.M., F.D., and A.K. prepared Tables 1, 2, 3, 4 and 5; Figs. 1 and 2. F.D. conducted the statistical analysis.

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Declarations

Competing interests
The authors declare no competing interests.

Institutional review board statement
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Informed consent
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